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Reducing *mass* confusion over the microbiome

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Abstract

Microbiomes harbor complex and diverse groups of microorganisms that together, either in a beneficial or detrimental manner, impact the physiology of their host and each other. As genetic tools continue to emerge and mature, more information is revealed about the identity and diversity of microbial community members. Genetic tools can also be used to make predictions about the chemistry that bacteria and fungi produce to function and communicate with one another and the host. Ongoing efforts to identify these products and link genetic information to microbiome chemistry rely on analytical tools. In this Tutorial, we aim to highlight recent advancements in microbiome studies driven by techniques in mass spectrometry. We focus on mass spectrometry due to its ability to identify a myriad of analytes from complex mixtures in both targeted and untargeted analyses. We discuss how different types of mass spectrometry workflows have supported recent microbiome studies in exploring the bacteria and fungi present in microbiotas, detecting their chemistry via targeted or untargeted approaches, and help understand how the microbiome-derived metabolites influence the physiology of living hosts and vice versa. We conclude with a discussion on the limitations and how they may be overcome with the development of both multimodal and multi-omics workflows as well as increased public accessibility to data to resolve the complexity of the microbiome.

Introduction.

Microbes evolved around 3 billion years before the first marine organisms existed.[3] Therefore, every other organism has co-evolved with or from microorganisms. Microbes often live among or inside these organisms, called “hosts” and often can influence the host’s overall physiology.[4] Microbes also coexist outside of a host as complex microbial consortia, such as lichen.[5] Microbiomes are comprised of communities of microbes that cohabit a specific environment and their biochemical properties.[1] Microbes within a a microbiome may interact by secreting biomolecules, including proteins, nutrients, or small chemicals. We will specifically focus on the small molecules in this outlook which have many names, including microbial metabolites, natural products, specialized metabolites, virulence factors, autoinducers, secondary messengers, or secondary metabolites. Broadly,

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microbial metabolites have functional groups and complex stereochemistry and serve important functional roles outside of primary metabolism, such as antibacterial agents like tetracycline or metal-scavenging molecules such as coproporphyrin III.[6]

There are many reasons why studying chemical interactions in a microbial environment is important. For example, by studying the metabolomics of microbial interactions, scientists can discover biologically active molecules with chemically diverse scaffolds that could be used for therapeutic purposes. Advanced understanding of microbiome chemistry can also lead to microbiome-derived personalized medicines or probiotic interventions. Additionally, gaining knowledge about environmental microbial communities can aid our understanding of chemical ecology and possible ways climate change can affect ecosystems. Microbiome science is rapidly moving beyond its nascency since technological advances in gene sequencing and mass spectrometry have facilitated unprecedented depth and coverage for identifying microbes and their metabolites from complex matrices such as fecal matter (Figure 1).

For the purposes of this Tutorial, microbiomes and their respective analytical methodologies, specifically mass spectrometry, will be discussed from two perspectives: host-microbe and microbiome community interactions. Host-microbe interactions involve the presence of a microbiota with a host organism, while community interactions focus on the chemistry between microbes. Metabolome interactions in both of these systems are complex, and the metabolites produced by these interactions often interfere or contribute to the host's metabolism.[7] Examples of these relationships include the human gut microbiome, the *Euprymna scolopes*- *Vibrio fischeri* symbiosis, and bacterial-nematodes such as *Wolchabia*-*Brugia malayi*.[8,9] However, there are also antagonistic host-microbe interactions, which include human pathogens such as *Staphylococcus epidermidis* and *Clostridium difficile*.[10,11] Recently, the FDA approved fecal microbial transplantation (FMT) as the standard of care for recurrent *Clostridium difficile* infections in the human GI tract.[12] FDA approval of FMTs comes after years of research providing direct evidence that the human gut microbiome possesses a range of defense mechanisms against pathogens through competition or chemical defense,with some researchers even considering the gut microbiome part of the immune system.[13] Furthermore, many recent studies show that diet can strongly influence the composition of the gut microbiome, and an imbalanced microbial composition could increase susceptibility to infections or parasites.[14] These examples highlight that the microbiome has the capacity to alter both community and host physiology.

Microbiome community interactions generally refers to microbe-microbe chemical communication and includes interactions between archaea, bacteria, fungi, phages, and viruses and their genes. Outside of the well-discussed human microbiome, there are critical microbiomes without organismal hosts that contribute to healthy ecosystems, such as within the soil or water in a lake. For instance, wildfires have a large impact on soil microbiomes, altering the ability of the microbiome to sequester carbon from the environment.[15] In addition, imbalances in the marine microbiome can be caused by increases in freshwater and nutrient runoff. These imbalances are associated with the rise in harmful algal blooms (HABs) that produce toxic microbial compounds, killing or harming marine life.[16,17] In these cases, researchers relied on a combination of

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genetics, metagenomics, and metatranscriptomics to identify members of these microbial communities, their functionalities, and their response to different environmental conditions. These approaches enable predictions about the chemistry that drives microbial colonization and function in their communities and with their hosts.

Previous approaches to measuring microbiome chemistry

In 1975, Anhalt and Fenselau applied pyrolysis-gas chromatography-mass spectrometry to demonstrate the presence of unique chemical signatures in extracts from different bacterial species, particularly phospholipids and ubiquinones (Figure 1).[18,19] Over time, other desorption/ionization techniques were introduced to develop modern high throughput microbial biotyping, such as the detection of proteins via matrix-assisted laser desorption/ ionization (MALDI) and electrospray ionization (ESI) (Figure 1). By 2009, instrumentation advances and developments in sample preparation also led to fungal identification (including yeasts, filamentous fungi, dermatophytes) with MALDI-TOF MS (Figure 1).[18] Coupled to these advances, bottom-up proteomics has become more accessible with the invention of the orbitrap in 2005 which aligns with the launch of the human microbiome project in 2007. In addition to the analytical advancements in resolving power, mass accuracy, throughput, and ease of use, biologists made advancements along a similar time scale in genetic sequencing in both humans and microbes. The increased affordability of genetic sequencing, highlighted by the invention of the nanopore sequencing device in 2014, is in direct contrast to the rising cost of mass spectrometers and their maintenance. Researchers have taken advantage of the advancement of both genetic and analytical technologies to characterize members of microbiomes in several contexts, with the first 16S rRNA sequencing of human fecal matter taking place in 1996 (Figure 1).[20] The simultaneous developments of sequencing technologies alongside advances in analytical instrumentation have enabled critical discoveries in microbiome science (Figure 1).

Current approaches in microbiome chemistry

Advancements in analytical chemistry have allowed scientists to better understand microbial metabolism, allowing for more precise measurements and metabolite identifications regardless of the sample's level of complexity. Recent developments in mass analyzers have resulted in increased mass resolving power with shorter duty cycles, which has resulted in an increase in both the number of metabolites detected and the ability to more confidently identify compounds (Figure 1). The introduction of ion mobility spectrometry (IMS) in 2011 has enhanced detection with the added collisional cross section (CCS) dimension's ability to separate isomers and isobars (Figure 1). Although these advancements have resulted in more features, defined as *m/z*-retention time pairs, identification of 2D and 3D structures remains a persistent hurdleDevelopment of MS/MS databases (GNPS in 2016 launched, Figure 1) and related tools has been revolutionary for microbial metabolomics, as scientists are able to more quickly identify compounds via MS/MS fingerprint and retention time matching.[21] These advancements have caused metabolomics to become a popular method for measuring microbiome-derived metabolites (Figure 1).

In addition, mass spectrometry imaging (MSI) can be used to spatially detect metabolites across a biological sample. For example, an intestinal sample can be prepared for imaging

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via the swiss rolling method, in which intestines, typically murine, are rolled up into a "swiss roll" spiral-like shape, flash frozen, and sectioned for imaging (Figure 2b).[22,23] Some microbiome metabolomics experiments involve orthogonal and complementary analytical techniques such as NMR;[24,25] there are additional considerations such as sample complexity and inherent limitations of relying on ^{1}H signals over a limited dynamic range, ^{13}C, or ^{15}N detection from limited microbial samples. Raman spectroscopy can provide additional information about microbial samples in a non-destructive, label-free manner that can complement other analytical methods (Table 1).[26] For the purposes of this tutorial, we will focus on mass spectrometry-based approaches, as they are most commonly used to probe microbiome chemistry; however, we recognize the importance of other analytical methods mentioned above, which are often used to answer questions that cannot be understood through mass spectrometry experiments.

Microbiome chemistry has been of particular interest to determine more precise functional roles of interactions within the microbiome and host. Studying the microbiome and the biomolecules associated with it, either as a whole or in reduced model systems, is analytically challenging as microorganisms interact with each other and with the host in a variety of nonlinear relationships.[27] In this Tutorial, we will highlight recent advancements in mass spectrometry challenges, and future considerations of separating, detecting, and characterizing metabolites that drive community and host-microbe interactions. Our focus will be solely on studies performed on bacterial and fungal members of microbial communities; archaea, viruses and phages are beyond the scope of this Tutorial.

General workflow

Getting started with microbial communities

Microbiome and host-microbe studies often begin by selecting a specific microenvironment of interest to design appropriate methods for subsequent measurements and analyses. For example, in the gut microbiota, researchers typically obtain feces noninvasively or intestinal fluids via biopsy or surgical intervention.[28] Surface sampling is also a common approach to probe a microbiome, and is often performed using a swab on samples such as human teeth or skin, with subsequent extraction. Biofluids, such as saliva, are also a common form of microbial sample (Figure 2e). Each of these sampling strategies yields a different biological matrix. The microenvironment dictates the nutrients and oxygen availability to the community which impacts the type of metabolites the microbes can produce. The microbial composition is then typically characterized by genetic sequencing technologies.[29] A common method of gene sequencing for the purposes of these experiments is 16S rRNA analyses, which does not require microbes to be cultured in order to identify them.[30] When relying on gene sequencing technologies, the steps taken during sample handling are crucial to the experimental design in order to avoid or account for contamination such as cross-contamination from samples in close proximity, the lab environment, or general sample degradation. Contamination of these samples can result in incorrect identifications, leading to false conclusions. Sample variability due to differences in microbial biomass across microenvironments may also impact results.[31]

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A major limitation of microbiome research is that many organisms cannot, as of yet, be cultivated in the lab. Best estimates suggest that only 1% of all bacteria have been cultured based on available 16S rRNA survey data.[32] Additionally, microbiomes consist of many members and designing multi species microbial community experiments is technically challenging due to different growth rates and nutrient requirements. Microbes that can be cultivated in the lab are supplemented with the necessary nutrients for growth, either in solid agar or liquid media, which enables direct measurements of metabolites from various members or community compositions. Growth media typically contains nutrients with a carbon and nitrogen source, minerals, and are buffered at a specific pH.[33] In the case of readily lab-cultivable species or simplified model microbiomes, such as the cheese rind model,[34] individual growth requirements like temperature or humidity can be controlled. Although there is no standardized media type for cultivating environmental isolates, there is evidence to support that even a small change in media components can have a significant effect on metabolite production.[35-38] However, in the current state of the field, this type of controlled cultivation inherently excludes microbes that cannot be cultivated but could be significantly contributing to the native microenvironment.

In order to address the complexity of microbial communities, there have been recent developments into creating model systems, specifically for building lab-amenable communities whose conditions can be altered in a controlled manner. Model systems have begun allowing researchers to probe the understanding of microbial communication, genetic regulation, and resistance. For example, the rhizosphere microbiome is known to contribute to plant health and disease and some microbial modifications improve agriculture yields.[39] Lozano et al. developed the Hitchhikers of the Rhizosphere (THOR), a model microbiome to study rhizosphere behavior by genetic analyses. This model consists of *Bacillus cereus, Flavobacterium johnsoniae,* and *Pseudomonas koreensis* grown on soybean exudate and includes some emergent properties of the rhizosphere, such as biofilm formation and colony expansion.[40] Emergent properties are derived from community interactions and cannot be predicted from the behavior of a single species.

Bulk community measurements

The majority of microbiome studies rely on untargeted metabolomics via LC-MS or LC-MS/MS to survey the small molecules found within the biological matrix; which typically involves a generalized workflow to capture as many metabolites as possible (Figure 2e). However, the chemistry captured during bulk extractions is biased at each step of the workflow given the choice of extraction solvents, solvent gradient, column phase, and instrument. Generally, each laboratory has unique protocols for extracting a broad spectrum of specialized metabolites, although there are efforts to standardize workflows, such as the National Institute of Standards and Technology (NIST) Metabolomics Quality Assurance and Quality Control (MetQual) program and Microbiome Quality Control Project (MBQC). NIST MetQual provides affordable homogenous QA/QC materials for laboratories to use and harmonize metabolomics measurements, while MBQC seeks to develop more rigorous protocols to improve experimental reproducibility.[41,42] Chemical extractions result in crude, complex mixtures containing biomolecules produced by the microbe. However, the extraction method biases the contents of the crude extract to include either more polar,

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nonpolar, or lipophilic metabolites depending on the solvents, thus some metabolites will remain undetected.[43] Following the generation of a crude extract, samples are typically analyzed via LC-MS/MS, gas chromatography-mass spectrometry (GC-MS), or ion mobility spectrometry-mass spectrometry (IM-MS) to separate the mixture constituents and collect structural information. More information on untargeted mass spectrometry experiments and the microbiome can be found in the review by Bauermeister et al.[44] Once specific knowledge of microbiome chemistry is known, targeted experiments to isolate, identify, or quantify the metabolite(s) can be employed.

Single cell and spatial community measurements

Generally, untargeted experiments rely on bulk measurements of the biological system to form a more targeted hypothesis. For instance, mass spectrometry imaging (MSI) provides information on the spatial distribution of metabolites and is compatible with measurements requiring cellular spatial resolution (Figure 2d).[45] We have comprehensively reviewed ionization source requirements for microbial and microbiome samples previously.[46] Briefly, in the case of most MSI experiments, thinly sectioned tissue samples containing microbes are often the ideal candidate for MALDI-MSI experiments, as they have a consistent sample height and need minimal or no drying time (Figure 2b).[47] However, whole organisms, such as Hawaiian bobtail squid hatchlings or symbiotic ants, and microbial colonies on agar medium can also be used to investigate spatial distribution of molecules (Figures 2a, 2d).[48-50] For commercial instruments that have fixed lasers or ionization sources, samples must also be flat.[48 51] MALDI-MSI provides an extra layer of complexity in comparison to other ionization sources like DESI, as the addition of a matrix can often impact the sample preparation protocol such as the drying time and temperature. Alternatively, LC-MS/MS extracts from distinct regions of the sample can be used to recreate low-resolution spatial mapping since MSI can be incompatible with some types of microbial samples.[52] Additionally, the incorporation of low-resolution spatial mapping can increase the depth of the metabolome detected, as MSI is prone to suppression effects, especially with the addition of a MALDI matrix. Computational methods have been developed to combat suppression effects through normalization.[53,54]

Single cell measurements, though technically challenging, are of importance moving forward, as metabolism across different cells throughout a sample are known to be heterogeneous.[55,56] Mass spectrometry and MSI are moving towards single cell measurements, however both instrumentation and data analysis pipelines are still actively in development in this emerging subdiscipline and compatibility with the typical size of a bacterial cell is challenging (~0.4 μm x 3.5 μm).[57] More information on microbial MSI can be found in Yang et al.[50]

Metabolomics data analysis

Once the data has been collected, the next step is typically to annotate the resulting data using public, vendor proprietary, or in-house databases to begin validating and testing the functional role of the annotated metabolite. There are many databases to choose from, however, it is these authors' observation that the Global Natural Products Social molecular networking (GNPS) has a high percentage of microbially derived tandem MS

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data in its spectral library and also allows for community contributions, which makes it highly relevant to microbiome research (Figure 3). GNPS employs spectral networking to match the experimental MS/MS data to query spectra using a cosine similarity score which takes absolute and relative mass shifts into account as well as fragment peak intensities.[21,58] An alternative approach can incorporate a paired omics strategy if the genome of the experimental organism is sequenced and publicly available. This type of approach allows for a comparison between what compounds the organism is predicted or known to produce versus what compounds are detected experimentally. Biosynthetic gene clusters (BGCs) for specialized metabolites can be putatively annotated using antiSMASH or fungiSMASH (Figure 3).[59] In the case of gut derived microbiota and other mammalian-derived microbiomes, gutSMASH queries primary metabolic gene clusters which encode taxon-specific, niche-defining metabolic pathways that are important to the host-microbiome interaction. These software rely on profile Hidden Markov Models (pHMMs), a probabilistic model which can capture the evolutionary changes to a related set of genetic sequences; gutSMASH is further distinguished by the use of taxonomic clade-specific pHMMs.[60] Additionally, Minimum Information about a Biosynthetic Gene cluster (MIBiG) contains user-input experimentally confirmed BGCs, and it is incorporated into AntiSMASH for comparison to known BGCs (Figure 3). The use of these *in-silico* tools can aid in identifying compounds produced by both single microbial cultures and complex cultures, understanding microbes' biosynthetic capabilities, and when used together can enhance our understanding of microbial communication, genetic regulation, and chemical defense. A discussion on the software available for preprocessing microbial mass spectrometry data and databases for *in-silico* dereplication can be found in the mini review by Grim et al.[61]

Microbiome studies

Replicating complex microbiomes in a lab-tractable system

Microbiomes are composed of many species of fungi, bacteria, archaea, and viruses; the human gut microbiome is estimated to contain approximately 100-1000 species.[62] Designing experiments to accommodate this degree of complexity remains daunting for testing hypotheses, thus the need for simplified model systems. Synthetic model systems are typically composed of members of the "core" microbiota with essential nutrients that mimic the habitat. Core microbiota are a set of microbes that are characteristic to the environment of interest based on metagenomic sequencing.[63] Abiotic factors such as temperature, humidity, nutrient availability, pH, salinity, and light can significantly alter how microbes interact and flourish with one another and their ecosystem. Synthetic models should reflect changes in biotic and abiotic factors across the experiment to be considered a lab tractable system. For instance, earlier we introduced THOR, since it's development , there have been a number of studies investigating the microbial community's robustness and resistance to these changes. Burman et al showed that small changes in temperature can greatly affect the composition of THOR and its ability to produce biofilms in comparison to the growth of its individual members.[64] This study can be applied to other model systems to better understand the effects climate change could have on microbial growth in agricultural microbiomes, as well as understanding the role microbes play in crop growth and health. Hurley et al probed the chemistry of THOR and revealed a major player in genetic regulation. Koreenceine,

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an antibiotic produced by *P. koreensis,* was found to be responsible for a large portion of differentially regulated genes in the other two community members.[65] Without the initial detection of this compound and its BGC, a significant factor in community survival and interaction would be overlooked. Thus, the development of model microbial communities like THOR paves the way for the development of new methods to understand microbiomes and their chemistry.

To begin synthetic model development, the known community should be dissected into individual species, from which their potential role in the microbiome may be deduced. The microbes are often grown as monocultures in liquid broth or solid media and analyzed. As ground truths of the system are established, the model system can be scaled up to include two, three, four, or more members. While some emergent properties can be captured and replicated, it is unlikely that all the emergent properties of the biological system can be replicated in the simplified model. However, poorly designed models that discount abiotic requirements needed for microbial growth on Petri dishes can have significantly altered chemical production by the microbes and the observed phenotype.[66]

Techniques in data acquisition

Due to the complex nature of microbiome samples, metabolomics of the model system often begins with bulk measurements to compare the individual metabolome to cocultures and community interactions. Putative identification of microbial metabolites by tandem mass spectrometry can be used to infer metabolic, signaling, and growth inhibitory pathways. Compounds of interest can be further isolated from extracts of large-scale growth of microbes on media and validated with 1- and 2D NMR experiments (Table 1).

MSI can be used for spatial mapping of putatively identified metabolites to the specific microbes. Imaging community samples can be performed by growing the microbes on agar and excising the sections containing the microbes and interaction zone for MSI (Figure 2d). This should be done across multiple replicates of the community to fully capture all the interactions. Alternatives to MALDI MSI that are common include DESI, nanoDESI, LAESI, or IR-MALDESI (Table 1). Putatively identified metabolites based on literature, database searching, or via tandem MS studies can be mapped spatially to the colonies, the interaction zone, or the agar. Therefore, an emergent metabolite from an interaction, such as an antibiotic, could be mapped to the specific microbe producing it and the microbe inhibited. Generally, the initial observations are validated using chemical complementation studies, e.g. purchasing or chemically synthesizing a standard, dosing it onto the agar, genetic knockout or complementation followed by examination of the phenotype or chemical profile from the microbes. A caveat to genetic validation is that the BGC must be known, and the BGC must be nonessential for survival. If the BGC is not known, there are often two methods to attempt to identify it: additional genome mining for potential proteins that could be involved in the biosynthesis or screening random transposons for metabolite production.[67,68] If the genotype is linked to the chemotype, further knockout mutants can be generated to examine the compound's biological significance. It is also important to note that many known compounds are not linked to any BGCs, and many BGCs

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are transcriptionally silent or quiet in laboratory settings (Figure 3).[69,70] The chemotype to genotype connection is still very much a challenge in this field.

Another important consideration for these studies is minimizing biomass that can impede ionization, especially for fungi. Recently, we have reported that the extraction areas are critical in enhancing microbial-derived features from fungi-bacterial interactions.[71] Alternatively, fungus can be cultured directly onto a nitrocellulose membrane (cellophane) and removed prior to MSI to image the remaining agar.[71,72] Culture time is another important consideration in microbial metabolomics experiments, as metabolite production is closely tied to growth rates.[73] It is likely that microbiome science will progress towards the study of community dynamics and assembly, building on early assessments of species diversity. Measuring the chemistry of these interactions will require the use of well-established synthetic microbiomes and standardized data acquisition procedures in mass spectrometry.

Host-symbiont Studies

Model host-microbe systems

Host-microbe systems are also challenging to study in the laboratory environment due to their complexity. Host-microbe systems often involve one to several microbes living in or on a host organism, such as humans, insects, invertebrates, or other animals. In order to study the interplay between multiple living organisms, scientists have developed several model host-microbe systems. For example, *Euprymna scolopes* (the Hawaiian Bobtail squid) and *Vibrio fischeri* (bacterium) symbiosis is an excellent model system, as it only involves one microbial symbiont which is easy to manipulate genetically. *V. fischeri* is the sole symbiont of the light organ and is selected by the squid from the water column upon hatching; this symbiosis is maintained for the squid's lifetime.[9] As a model system, *V. fischeri* uses biofilm formation to assist in its colonization of epithelial tissue, which closely mimics the pathogenic colonization of *Vibrio cholerae* and *Pseudomonas aeruginosa* in human tissues such as the gut or lungs.[74,75] Although colonization and bioluminescence have been thoroughly studied, the chemical communication related to colonization and maintenance within the host are still unfolding.[76,77]

Studying chemical communication in model systems can allow scientists to develop mass spectrometry methods to study pathogenic systems without the risk of working with higher biosafety level organisms. These methods can then be applied to pathogenic systems and even additional non-pathogenic systems, such as the human gut microbiome, to better understand how microbial metabolites can interact with human metabolism.

Techniques in data acquisition

It is important to note that oftentimes, host-microbe studies involve both bulk and spatial measurements, depending on the hypothesis being tested. If the goal is to detect novel metabolites that may be differentially regulated in specific genetic or growth conditions or without the need to specifically identify the producing organism, bulk measurements can be effective following solvent extraction. For example, some species of ants such

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as *Apterostigma dentigerum* cultivate fungus for food (cultivar). However, the parasitic fungus (*Escovopsis* sp.) can damage the cultivar. *A. dentigerum* has symbionts such as *Pseudonocardia* spp. and *Streptomyces* spp. that produce antibiotics to protect the cultivar fungus from infection. LC-MS was employed to discover that dentigerumycin was a major metabolite in a co-culture of these organisms. Furthermore, dentigerumycin produced by *Pseudonocardia* was used to defend the cultivar from the parasitic *Escovopsis.*[78] In this case, LC-MS was central to the discovery of a metabolite that played a role in this symbiotic relationship.[79]

Additionally, the spatial distribution of molecules across solid microbial samples can be important to understand when testing a hypothesis. In these instances, spatial measurements using MSI can be helpful, as even microbial monocultures can have heterogeneous distribution of metabolites.[80,81] Spatial measurements have also been collected using MSI for many whole organisms, such as *V. fischeri*-colonized squid, plant-microbe system *Ardisia crenata*-candidatus *Burkholderia crenata*, and ant-microbe systems (Figure 2a).[76,82] This method was applied to the aforementioned ant-fungal-cultivar relationship, where another potent antibiotic, valinomycin, was found to be produced by symbiotic *Streptomyces* spp. living on the ant's surface. Valinomycin was found to be centered around the joints of the ants' legs, which provides evidence that *Streptomyces* spp. don't necessarily produce this antibiotic to protect the cultivar from *Escovopsis* spp.; however, it likely protects the individual ants from pathogens and parasites. Although these spatial studies provide additional biological context, it is still unknown how and if the ant workers can regulate the production of antibiotics like valinomycin.[83 49]

The majority of host-microbe system studies, such as the examples above, involve a combination of untargeted LC-MS/MS metabolomics and one of the many MSI methods (Figure 2d). The ability to use different sources or instruments to analyze samples can sometimes result in a more comprehensive dataset due to differences in ionization between instruments.[84] For instance, ESI-LC-MS/MS is often used to first identify possible compounds of interest in a host-microbe system. Then, MSI can be used to view the spatial distribution of the compounds of interest to further understand the biological context of the metabolites detected. Both methods provide integral information in understanding microbial communication in host-microbe systems.

Future outlook

Using multiple mass spectrometry techniques to probe microbiome and host-microbe research

The study of microbiome-derived chemistry and host-microbe interactions have advanced significantly due to improvement and accessibility in analytical methodologies and instrumentation that have coincided with advances in gene sequencing. Specifically, mass spectrometry-based metabolomics has emerged as a robust tool for identifying and characterizing microbial metabolites. By employing MS, scientists can begin to elucidate the functional role of microbiome interactions within communities and in host-microbe relationships. However, it is critical to recognize that no single MS technique can provide a comprehensive sampling of the metabolome. Bulk measurements can be applied to

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both microbial colonies and microbe-containing tissue samples, as they both require extraction, and they can offer information on upregulated metabolites in many different biological contexts (Figures 2b, 2c, 2d, 2e). Additionally, spatial measurements can allow visualization of spatial distribution of metabolites in multiple sample types, providing additional biological context that cannot be understood through bulk measurements (Figures 2a, 2b, 2c, 2d). When combined, bulk and spatial measurements can complement each other and can better unravel the complex network of microbial interactions. A multi-faceted approach to microbiome science provides valuable insight into the underlying chemical mechanisms of many key interactions.

Challenges to be addressed in order to advance microbiome and host-microbe mass spectrometry research

There are some challenges that must be overcome to maximize the potential of MS in microbiome science. First, model systems may not always accurately represent a microbial community, although synthetic models are critical to the development and application of MS technologies. For example, non-lab cultivable organisms and their chemistry are naturally excluded from model systems. Environmental microbes and their metabolites can be measured with a simple swab of the area they live in or on, but if they can not be cultivated at scale, the isolation and elucidation of unknown compounds through MS or NMR becomes difficult. In addition, cultivation of host organisms also remains a hurdle. For instance, sea sponges and their microbiomes produce very unique chemistry, but they are difficult to cultivate due to their sensitivities to pH, temperature, and nutrient sources.[85] These biological factors remain a significant challenge in the field.

Another major impediment to microbiome research is potentially undersampling of metabolites due to either sample preparation methods or ionization. The type of metabolites detected in LC-MS(/MS) is dependent on extraction methods and ionization sources. Oftentimes, fractionation by polarity prior to LC is performed using solid phase extractions (SPE).[86] However, these fractionation methods often use mostly polar solvents such as water and methanol. Assuming the analyte of interest is a polar molecule, nonpolar and lipophilic metabolites may be excluded to some extent, and vice versa. Additionally, in untargeted approaches, it is typically more commonplace to run experiments in positive mode, as more biomolecules ionize at a higher intensity in positive mode.[87] Negative mode experiments tend to only be incorporated if the researcher is seeking to identify metabolites that ionize better in negative mode, such as lipids or phosphorylated metabolites. In any ionization source, ionization efficiency of the analyte is a persistent limitation–a major assumption in these experiments is that the most abundant metabolites in these microbiomes are ionized. Additionally, even ions that do ionize at low intensity may not be fragmented given the stochastic nature of data dependent acquisition, which is a popular acquisition mode in untargeted metabolomics research. If untargeted analysis is done via MALDI, the choice of matrix influences the ionization of specific analyte classes.[88] In any case, using ionization as a means to measure metabolites in general will inherently overlook molecules that do not ionize well under any condition and may be playing an important role in the microbiome.

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Standardizing workflows in microbiome research is critical to ensuring consistent and reproducible results, facilitating data sharing and collaboration, mitigating potential bias and errors, and increasing overall scientific rigor of results. Even with standardized procedures, experimental results can vary based on instrument type, proximity to maintenance date for the instrument, ambient weather, and so on. This is a recognized challenge in microbiome research that several initiatives seek to address, such as the previously mentioned NIST, MetQual, and MBQC.[41,42] However, in the current state of the field, a majority of labs continue to use in-house metabolomics protocols, as no one method has been widely adopted.

Once the experiment is completed, processing and analyzing the data presents another hurdle. To start, making data publicly available is not yet a requirement for publication in many cases, causing most data to be kept within a lab, which makes comprehensive database searching incomplete. There have been many open-access softwares and R packages introduced for mass spectrometry data processing and analysis, but many are not maintained as fast as their dependencies are updated.[89] Additionally, vendor proprietary or paywalled software can be a financial burden for small academic labs and researchers will often resort to in-house workarounds, which are often non-standardizable. To combat the historical lack of data availability and unequal access, the microbial science community has come together to create free, public databases, composed of contributions from the community. [29] Some powerful tools and databases have been introduced by the community as open-access, such as GNPS,[21] AntiSMASH,[59] and NPAtlas[90] are routine to many workflows (Figure 3). Ongoing efforts to develop comprehensive software and Findable, Accessible, Interoperable, Reusable (FAIR) databases[91] will greatly enhance the ability to study microbiome chemistry and will continue to only be effective with contributions from the whole community.[92]

Concluding remarks

While there are challenges to overcome, applying analytical methods to the study of microbiome chemistry and host-microbe interactions has already yielded significant findings. Parallel advances in mass spectrometry and microbiome science have revolutionized the ability to measure the chemistry of complex systems (Figure 1). Continued advancements in analytical methods will lead to better separation, detection, and characterization of metabolites that are critical to new insights in microbiome research. Finally, the democratization of the data will positively impact our ability to move towards functional annotations and studies (Figure 3).

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Biographies

Dr Laura Sanchez pursued a PhD in Chemistry at the University of California, Santa Cruz (UCSC), and was a NIH K12 IRACDA postdoctoral fellow at UC San Diego. She started her independent career at the University of Illinois at Chicago in the Department

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Casey Heaney pursued a Masters of Science in Chemistry at UCSC and was an NIGMS-IMSD fellow. She was a member of the Sanchez lab where she used mass spectrometry to study fungal-bacterial interactions derived from the cheese rind microbiome.

Allyson McAtamney is pursuing her PhD in Chemistry at the University of California, Santa Cruz in Dr. Laura Sanchez's Lab, where she studies chemical communication in host-microbe systems. Previously, she earned her BS in Chemistry at Marian University Indianapolis.

Itzel Lizama-Chamu earned her BS degree at the University of Illinois Chicago where her research focused on adapting mass spectrometry to search for bioactive natural products produced by cheese-rind fungi in Dr. Laura Sanchez's lab. She is currently pursuing her PhD in Chemistry at the University of California, Santa Cruz in Dr. Laura Sanchez's lab and is an NSF-GRFP Fellow.

Common Terminology

Microbiome

A community of microorganisms and entities (e.g. fungus, bacteria, archaea, protists, viruses, phages) and their biochemical activity in a certain environment.[1,2]

Microbiota

All living microorganisms (e.g. fungus, bacteria, archaea, protists) in a certain environment.[1]

Community

All observed entities and their interactions (e.g. fungus, bacteria, archaea, protists, viruses, phages) in a certain environment.[2]

Microbiome community interactions

Biochemical interplay between microbiota mediated by metabolites and gene transfer.[1,2]

Host-microbe interactions

Biochemical interplay between the host and microbiota mediated by metabolites and gene transfer.[1]

Microbial metabolite

Metabolites that are biosynthesized by microbes. May also be referred to as specialized metabolite, secondary metabolite, natural product, and biomolecule.

Model system

Simplified experimental strategy which captures key taxa and/or host for the study of host-microbe and community interactions.

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Metabolome

For the purpose of this tutorial, the metabolome will be defined as microbial metabolites, as well as primary metabolites produced, consumed, and/or modified by the microbe.

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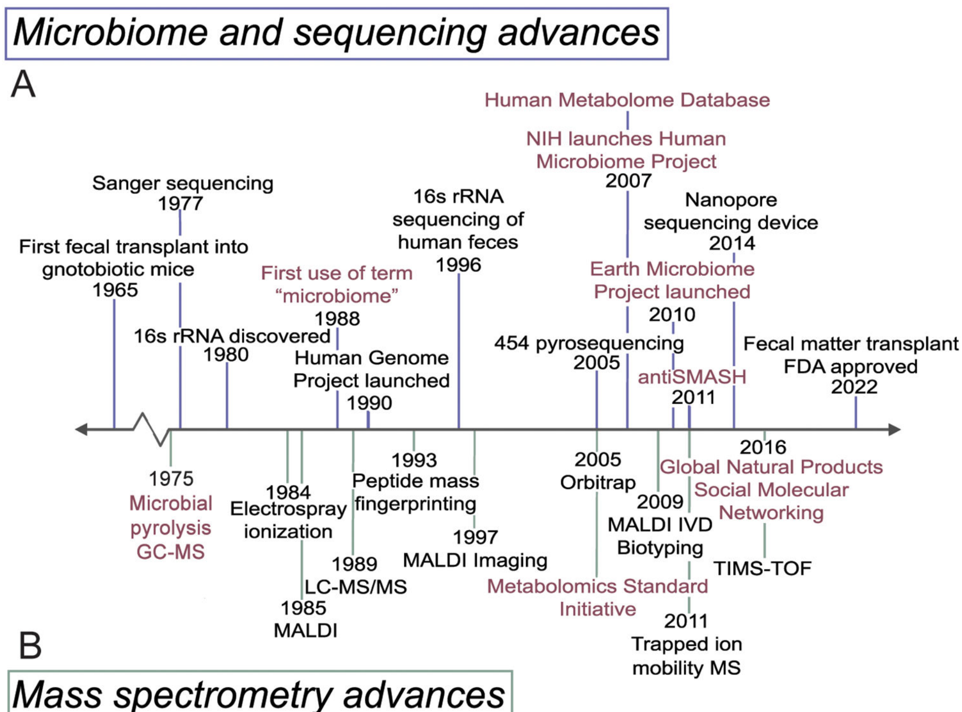

Figure 1:

Timeline of some major technological advances in (A) Microbiome science[12,105-108] and (B) Mass Spectrometry adapted from Palmblad et al.[109] Red font corresponds to advances in metabolomics research.

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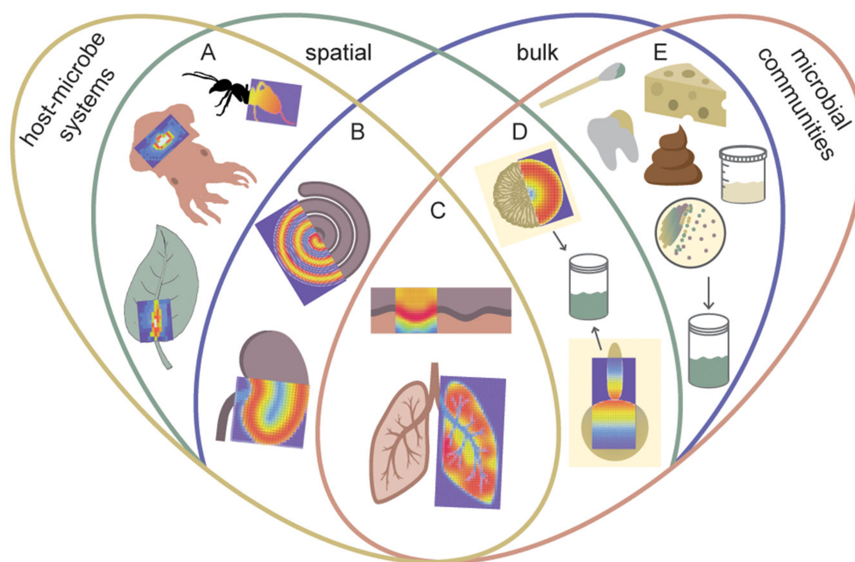

Figure 2:

Host-microbe vs. microbiome approaches based on sample type. (A) Spatial interrogation of host-microbe systems often involve whole organisms, such as plants, insects such as leaf-cutting ants, the Hawaiian bobtail squid and their associated microorganisms.[48,82,83] The samples can be directly collected from the native environment or lab cultivated. (B) Larger organisms that serve as model systems for humans, such as mice, facilitate measurements of organs that can be cryosectioned for spatial or extracted for bulk analysis of microbial metabolites in that organ. Examples of this include swiss rolled murine intestines and spleen infected with pathogenic bacteria.[51,110] (C) Human tissue samples that have been donated for scientific research or biopsies from living patients can be used for either spatial or bulk analysis to interrogate the interface of the host and microbial communities. There are limited examples of this due to the complexities and consent required to collect these samples.[52,111] (D) Microbial cultures, whether monoculture or complex community, can be analyzed spatially to gain metabolomic information about heterogeneity throughout the culture. These samples can also be extracted and analyzed for presence/absence of metabolites without spatial information.[6,81,95] (E) Some samples are only compatible with bulk analysis of metabolites due to their complexity. For example, human microbial communities can often only be interrogated via surface sampling using a swab, then cultured and extracted. Biofluids are also incompatible with spatial analysis. This is where a majority of known methods to study microbial communities lie.[23,93,112] A benefit of this type of non-invasive sampling is that it can be compatible with longitudinal study design.

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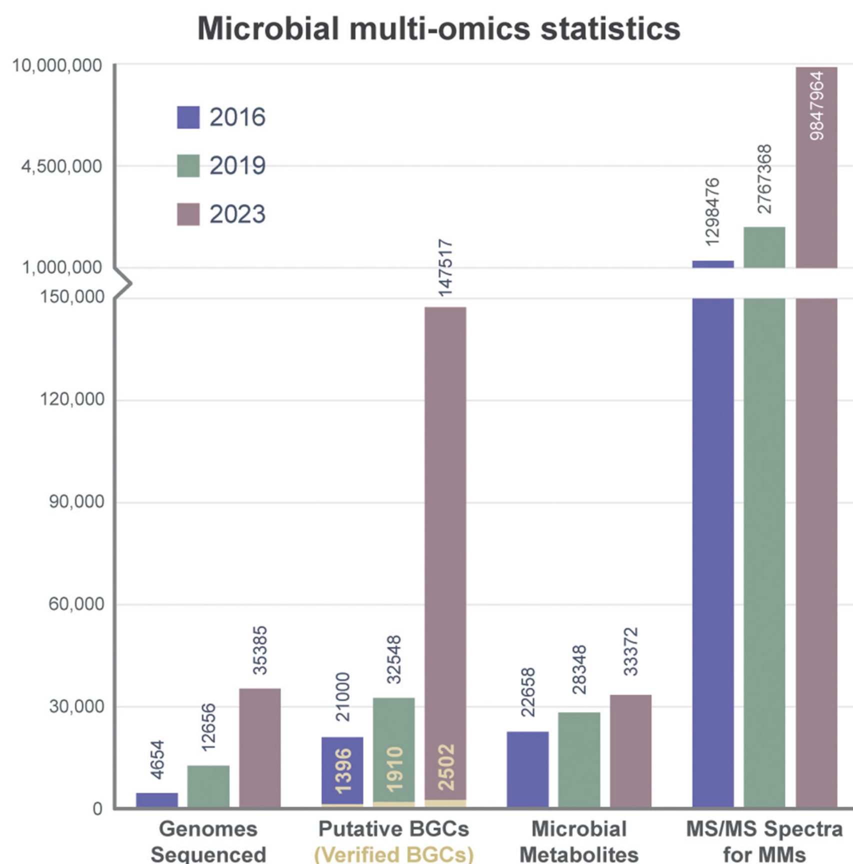

Figure 3:

Column chart of genomic and metabolomic statistics from open-access sources by year (2016, 2019, 2023). All 2023 data was accessed on May 16, 2023. 2016 and 2019 statistics are based on the number of entries at 12:01 am on January 1st of that calendar year, unless otherwise stated. The number of genomes sequenced was obtained using NCBI and recording the number of complete microbial genome assemblies only. Putative BGCs were found using the AntiSMASH database with 2016 and 2019 statistics acquired from publications for versions 1.0 and 2.0.[113-115] Verified BGCs represent the number of experimentally characterized gene-metabolite connections, downloaded from MIBiG versions 1.3 and 2.0.[116] The number of microbial metabolites was obtained from a download of the entire NPAtlas database.[90] A search of the MassIVE database was performed by filtering either the "Title" or "Keywords" fields for "microb" and manually assessing the results for only microbial metabolomics datasets. It is important to note there is likely redundancy in these datasets, as there can be multiple adducts and MS/MS spectra for any analyte as well as multiple instances across different acquisitions.

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Table 1:

Table of various metabolomics-related experimental techniques and their general purpose, advantages, potential limitations, and references to exemplary studies.

		Experimental Focus:				
Detection Method	Experiments	Host-Microbe	Microbiome	Advantages	Limitations	References
Mass Spectrometry	LC-MS/MS	Bulk measurement, low-resolution spatial mapping, structure elucidation	Bulk measurement, low-resolution spatial mapping, structure elucidation	Small sample quantity needed, chromatographic separation improves spectral deconvolution	Must be soluble in organic solvent, extraction method biases measurement	52,78,83,93
	MSI	High-resolution spatial mapping, bulk measurement	High-resolution spatial mapping, bulk measurement	High-resolution imaging for samples of varying size	No live cell analysis, must withstand prolonged drying, low ionization efficiency	6,22,49,76,81,82,94,95
	DESI	High-resolution spatial mapping, bulk measurement	Map community interactions, Bulk community measurement	Little to no sample prep required	Low spatial resolution and solvent dependant	23,72
NMR	1D and 2D	Detecting specific changes across conditions	Detecting specific changes across conditions	Highly reproducible, minimal sample preparation, structure elucidation	Samples generally not enriched in spin-active nuclei required for high-sensitivity experiments , spectral overlap challenges deconvolution, larger sample quantity needed	24,25,96-98
Raman	Spontaneous	Map host-microbe interactions in live cells, biomarker detection	Map community interactions, bulk community measurement, species identification, biomarker detection	Relatively inexpensive, reagent-free, nondestructive, single cell analysis	Can only provide general information about macromolecules, relatively weak signal	99-102
	Surface enhanced (SERS)	Map host-microbe interactions in live cells, biomarker detection	Map community interactions, bulk community measurement, species identification, biomarker detection	Enhanced signal intensity, nondestructive, reagent-free, single cell analysis	Can only provide general information about macromolecules, sample must be applied to surface, costly substrate	103,104