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Emerging Technologies for Gut Microbiome Research

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Abstract

Understanding the importance of the gut microbiome on modulation of host health has become a subject of great interest for researchers across disciplines. As an intrinsically multidisciplinary field, microbiome research has been able to reap the benefits of technological advancements in systems and synthetic biology, biomaterials engineering, and traditional microbiology. Gut microbiome research has been revolutionized by high-throughput sequencing technology, permitting compositional and functional analyses that were previously an unrealistic undertaking. Emerging technologies including engineered organoids derived from human stem cells, high-throughput culturing, and microfluidics assays allowing for the introduction of novel approaches will improve the efficiency and quality of microbiome research. Here, we will discuss emerging technologies and their potential impact on gut microbiome studies.

Keywords

Gut Microbiome; Automated Culturing; High-Throughput Sequencing; Personalized Medicine; Microbial Cultivation

The Gut Microbiome

Humans are associated in a symbiotic relationship with up to 10^{14} microorganisms [1]. The majority of these host-associated microbes reside within the gastrointestinal tract, and have extraordinary metabolic potential, playing a pivotal role in human health [2]. The gut microbiota enhances the host's response to pathogen invasion [3], modulates host gene expression and immune response, and ultimately impacts overall health [4-6]. Normal inhabitants of the gastrointestinal tract facilitate the metabolism of polysaccharides consumed by the host [7] and interactions between microbes within the microbiota enhance this metabolic potential, further improving polysaccharide utilization [8]. Other health-supporting functions of gut microorganisms include disease protection through

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immunomodulation [9] as has been shown for *Bifidobacterium longum,* which strongly stimulates production of interleukin-10 and proinflammatory cytokines including TNF-α [9] that protects the host against tumor proliferation [10]. Furthermore, *Bifidobacterium* and other lactic acid bacteria often produce exopolysaccharides (EPS), complex polymers that can be used as fermentable substrates by other gut microbes aiding in microbial community structure and stability [11].

Compositional perturbations of the **microbiota** (**dysbioses**) (See Glossary) have been associated with diseases including obesity [12], diabetes [13], colorectal cancer [14], and allergies [15]. Hence maintaining compositional and functional stability within gut **microbiome** is essential to host health as demonstrated by dysbioses detected at the onset of nonpathogenic chronic diseases [4, 5] like Crohn's disease, where the gut microbiome had a significant decrease in beneficial Bifidobacteriaceae populations while exhibiting an increase in groups containing potential pathogens including Enterobacteriaceae, Pasteurellaceae, Fusobacteriaceae, and Neisseriaceae [16].

Current high-throughput sequencing technologies provide important information about the composition and functionality of the gut microbiome. However, in order to better understand mechanistic interactions between the gut microbiota and its host, and the importance of the gut microbiome in maintaining health, it is critical to explore new research approaches and integrate emerging technologies from multiple branches of biology and engineering [17]. The present review will discuss recent breakthroughs in microbiome studies and biotechnological advancements that may improve our ability to study the gut microbiome in the future.

The Study of the Gut Microbiome Before Next-Generation Sequencing (NGS)

Prior to the advent of NGS, the accurate identification of most members of a complex microbial community was challenging. This was especially true for the gut microbiome, a highly diverse community and one of the densest microbial communities on the planet, with a small percentage of culturable microbes [18]. Early gut microbiome studies involved cultivation of individual bacteria [19], and studies of interactions by co-culture of microbial consortia [20]. While these methods allowed investigation of basic microbial interactions, they provided little information about community dynamics, as uncultivable microbes were excluded from analysis and organisms were outside of their natural environment. Cultivation based approaches to study microbial communities are still important today; however, new techniques have emerged over time enabling the study of communities in greater detail without omitting uncultivable microbes. Early methods to determine microbiome composition allowed the differences between microbial communities to be discerned, but offered little information regarding taxonomic composition of communities. Terminal-restriction fragment length polymorphism (T-RFLP) for example was used to identify gut dysbioses associated with necrotizing enterocolitis (NEC) in animal models, suggesting a significant role for *Clostridium* in pathogenesis [21]. Similarly, a **denaturing gradient gel electrophoresis (DGGE)** study showed that the human gut microbiome was disrupted by

antibiotic administration but the impact was variable between patients, consistent with data obtained by culture-dependent methods [22]. Finally, **temperature gradient gel electrophoresis (TGGE)** showed a direct correlation between *Sphingomonas* with NEC in human infants, and associated antimicrobial treatments with significantly reduced gut microbial diversity [23].

As DNA sequencing technology emerged, the ability to study the composition of complex microbial communities improved, allowing for more precise and rapid taxonomic identification of individuals within those communities. Early sequencing technologies were slow and expensive. Sequencing analysis of the **16S rRNA** gene was originally performed by cloning the full gene into plasmid vectors, transforming it into suitable hosts (usually *Escherichia coli*), and sequencing it [24]. Due to the cost of sequencing at the time, other methods like Southern blotting and *in situ* hybridization made use of the 16S rRNA clone libraries to identify members of complex microbial communities [25].

More recent PCR-based massive parallel sequencing technologies have drastically increased throughput of microbiome analyses [26]. With the ability to enrich templates with initial concentrations too low to detect, PCR based sequencing technology allows identification of individuals with very low populations within complex communities. PCR-based sequencing strategies along with advancements in the availability of bioinformatics tools opened the door to modern day NGS technology.

While advanced bioinformatics platforms for data analysis provide an invaluable tool for microbiome research (Box 1), vast amounts of data obtained through high throughput sequencing far surpasses the ability to analyze that data, creating a bottleneck in interpretation of results. As new bioinformatics tools are designed, more information will be derived from data obtained from high throughput sequencing platforms.

NGS Technologies and Metabolomics in Gut Microbiome Research

Connections between microbes and host health were postulated before the advent of high throughput sequencing technology [27]. However, prior to technological advancements, these studies lacked the ability to take entire community dynamics into consideration. The identification and relative quantification of microbial taxa via high-throughput sequencing drastically improved the ability to study the gut microbiome. While initially focused on compositional studies (who is there?), microbiome researchers became increasingly interested in determining functionality (what are they doing?) and identifying modulators and mechanistic networks within complex microbial ecosystems (how do microbes interact?) (Figure 1).

Today, one standard method for determination of gut microbiome composition is performed by isolation of total DNA from samples, PCR amplification of regions within universally conserved 16S/18S rRNA genes, followed by high-throughput sequencing of those amplicons. This technology has eliminated the need for cloning individual genes, blotting for specific RNA, or cultivating individual microbes to identify members of a community. High-throughput amplicon sequencing has generated a wealth of data on microbiome

composition from different environments and conditions including the gut microbiome of ~1000 year old preserved human remains, which showed that, similar to modern day humans, Firmicutes was the dominant bacterial phylum within the gut at that time [28]. The study of the gut microbiome throughout history may give some insight into the differences between modern humans and our ancestors, and provide new understanding of diseases that have arisen more recently in history. In 2012, the Human Microbiome Consortium (HMC) published reference metagenomes of microbes present within healthy humans. Although HMC data, obtained predominantly by use of 16S rRNA sequencing and Quantitative Insights Into Microbial Ecology (QIIME) [29] analysis, showed that the dominant microbial taxa in the human gut include Bacteroidetes, Firmicutes, and Proteobacteria, and that species including *Bacteroides fragilis*, *Bacteroides melaninogenicus*, *Enterococcus faecalis*, and *E. coli* are present in the majority of healthy human subjects [30] (Figure 1), other studies have suggested that no bacterial species is present within 100% of subjects [31, 32]. It is important to note that taxonomic characterization of the microbiota does not always translates into function, despite the fact that compositional changes often do result in functional changes. In order to effectively understand the impact of the microbiome on the host, it is critical to connect compositional to functional studies by combinations of NGS technology and functional microbiological assays.

NGS technology has provided the opportunity for studies focusing on complex microbial systems to be performed more effectively than ever before. However, biases introduced by PCR based sequencing platforms can skew results, over or underestimating bacterial abundance [33, 34]. When performing 16S rRNA amplicon sequencing, primer efficiency, PCR amplification conditions, sequences platform, bioinformatics pipeline, and protocols for DNA extraction and sample handling can all introduce biases to results [32]. The Microbiome Quality Control project (MBQC), a consortia of scientists aiming to standardize microbiome studies, has shown that difficulty in producing reproducible results in microbiome studies are largely a consequence of these internal biases [35].

Whole genome shotgun (WGS) sequencing extends the information provided by 16S/18S rDNA amplicon sequencing allowing the identification of DNA viruses and providing information about gene content and metabolic pathways [36]. The gut virome is dominated by bacteriophages (predominantly bacterial DNA viruses), but includes a diverse population of both DNA and RNA encoded eukaryotic viruses [37, 38]. The virome has an important role on host health by modulating the bacterial community, as well as by interacting directly with host cells [37, 39]. However, virome data is often omitted from microbiome compositional studies, as currently most studies are based on 16S/18S rRNA amplicon sequencing data. Bacteriophages can modulate composition of the microbiome by killing their bacterial hosts during their lytic growth or by altering gene expression during lysogenic conversion [40]. Additionally, eukaryotic viruses and bacteriophages in the gut have been shown to encode genes involved in DNA replication, amino acid, lipid, and carbohydrate metabolism, signal transduction, and transcription regulation [37, 38].

By gaining information about all DNA present, it is possible to infer functionality of microbes within the community by comparing identified genes and predicted proteins from sequence data to databases including the Kyoto Encyclopedia of Genes and Genomes

(KEGG) [41]. In a recent study, WGS in combination with 16S rRNA sequencing showed significant differences in diversity and species richness between the gut microbiome individuals of different ages and ethnicities. Functional analysis from WGS data showed that the microbiota of children and adults differed in the abundance of genes involved in amino acid metabolism, lipopolysaccharide (LPS) biosynthesis, RNA degradation, and steroid hormone biosynthesis, suggesting differences in the functional potential of the microbiota [42]. While a great deal of information can be obtained through WGS, a drawback is that the generated functional information represents only potential functions since genes identified may or may not be actually expressed within the community under the studied conditions.

As sequencing technology has improved, it has become possible to better study the activity of microbes within complex communities. RNA sequencing (RNAseq) permits the analysis of gene expression adding valuable expression data to compositional data sets [43], as well as providing compositional data for active microbes within the community [44]. **Metatranscriptome** data from the gut microbiome has shown that nearly 50% of genes actively expressed are involved in transport and metabolism of carbohydrates, amino acids, nucleic acids and lipids, as well as energy production and conversion, indicating that the microbiota enhances the metabolic potential of the host [44]. Transcriptome data can be used to explore the effects of perturbations and environmental factors on the function of the gut microbiome. Furthermore, transcriptome data can identify microbiome functional change before a compositional change occurs [45, 46]. For example, bacterial ribosomal proteins are generally overexpressed in a healthy microbiome [45] and a decline in ribosomal protein expression can occur when microbial growth rates begin to decline, but prior to a change in the microbial population. This could serve as a diagnostic tool to detect signs of dysbiosis prior to the onset of disease. Finally, transcriptome data can provide information about bacteriophage prevalence and activity within the community, which is often overlooked [47].

In addition to using RNAseq to understand microbe-microbe interactions and microbial activity within the gut, transcriptome data collected from host cells provide information regarding interactions between host and microbiome. Recent studies have shown that the microbiota directly alters the transcriptome profiles of its host, with as much as 10% of the transcriptome being regulated by the microbiome [48]. However the mechanisms by which this transcriptome modulation occurs is largely unknown.

Understanding of the entire pathway from gene sequence to function is completed by **metabolomics** analyses of gut samples. Identification of metabolites produced within the gut microbial community provides a detailed picture of functionality and a better understanding of physiology. Most microbial metabolic processes like carbohydrate fermentation generate byproducts that impact the community as well as the host. One example is the fermentation of carbohydrates into short-chain fatty acids [49], which act as signaling molecules to both host and other microbes [50, 51]. Traditionally, mass spectrometry (MS) and nuclear magnetic resonance (NMR) have been the primary tools used in metabolomics analyses. Advancements to those technologies, including Matrix-Assisted Laser Desorption Ionization time of flight (MALDI-TOF), Secondary Ion Mass Spectrometry (SIMS), and Fourier transform ion cyclotron resonance MS have improved the throughput and accuracy of metabolomics studies [52-54].

Advancements in Culturing Technologies

The information provided by compositional and functional studies of the gut microbiome have permitted inference of microbial interactions within complex communities. After determining the composition of the gut microbiome, and the microbial genes and pathways impacted by health or disease condition, the next logical step is to address the mechanistic networks involved in microbiome-host interactions. In order to explore the mechanisms by which individual microorganisms modulate the microbiota and host physiology, they must be identified, isolated, and studied in pure cultures and microbial consortia. Unfortunately, a relatively small percentage of microbes that reside within the gut are **cultivable**. Although recent studies using gnotobiotic mice and anaerobic culturing techniques were able to successfully culture ~50% of bacterial species identified by 16S rDNA amplicon sequencing, covering nearly 70% known genera and >90% families [55, 56], most of the diversity present in the gut microbiome is at the strain level making identification and cultivation a difficult task.

Live gnotobiotic animals provide novel methods for cultivation of difficult to grow microbes [56], and to study gut microbe-host and microbe-microbe relationships [57]. Colonization of gnotobiotic mice with defined gut microbial consortia showed that bile acids were significantly reduced in mice colonized with a community containing *Bacteroides ovatus*, while plant-derived quinic acid reached its highest levels when *Odoribacter splanchnicus* was present but *E. coli* was absent [57], suggesting microbe-specific interactions were modulating the host's metabolic potential. Furthermore, humanized gnotobiotic animal models have been instrumental in understanding the effects of the human gut microbiota on its host. By colonizing germ-free mice with healthy human gut microbiota via fecal microbiota transplantation, researchers showed that humanized mouse gut microbiota successfully transferred from generation to generation without loss of microbial diversity [58] resulting in a method to grow and study human-associated microbes within gnotobiotic animal models.

Advances in culturing technology including use of anaerobic conditions and gnotobiotic animals have advanced traditional microbiology approaches, as many previously uncultivable microbes can now be cultured in lab settings designed to imitate their natural growth conditions, allowing the isolation of previously uncharacterized species [59]. A device specifically designed to identify uncultivable microbes within complex microbial ecosystems is the isolation chip (iChip). iChip consists of a chamber containing multiple cells that isolate microorganisms within a mixture while allowing for passage of nutrients [60]. While iChip was intended for drug discovery and identification of microbes in environmental samples, the technology has the potential for isolation of uncultivable gut microbes. A derivative of this technology (I-tip), which uses the same premise as iChip, trapping individual microbes within a gel that allows for passage of metabolites and nutrients, but at a smaller scale, has already been applied for *in situ* isolation of microbes from invertebrate organisms yielding isolates from 34 novel microbial species across 5 major phyla [61].

New culturing technologies and the information provided by NGS have together made high-throughput culturing possible [59, 62]. Growing individual community members at the same time under automated conditions permits a more rapid and thorough study of a community as a whole. By utilizing a combination of **microfluidics** and fluorescence-activated cell sorting (FACS) technologies, it has become possible to isolate and culture microbes based on the generation or consumption of metabolites in a high-throughput fashion [62] using fluorescent reporters for specific molecules to screen diverse microbial populations for individuals producing metabolites or enzymes of interest, or screening for mutations in known pathways.

Moreover, advancements in culture control technologies have made it possible to simulate gastrointestinal (GIT) conditions such as acidity and bile concentration [63]. The simulator of human intestinal microbial ecosystem (SHIME) has been used to establish stable, reactor-grown gut microbial communities. SHIME uses a series of linked reactors each of which simulates a different region of the human gastrointestinal tract. When colonized by microbes, each region of the simulator has a unique microbial community, similar to the living host, including the prevalence of *Bacteroides/Prevotella* and *Lactobacillus* in the 'colon' [64]. Similarly, Robogut has been used for *in vitro* cultivation [65] to produce microbial stool substitutes for use in treatment of *C. difficile* infections [66].

Finally, new microscopy and imaging technologies have a remarkable importance in microbiome research [67], permitting direct observation of microbial colonization, location, and microbial and host-microbial interactions in the intestine. In a recent study, immunofluorescence images of gut cross sections were used to demonstrate how dietary changes directly impact host-microbe interactions. Specifically, elimination of microbiota-accessible carbohydrates from the diet increased the proximity of microbes to the epithelium, increasing expression of the inflammatory marker REG3β [68].

Use of *in vitro* systems to study gut microbial ecology is a useful tool to study the effects of perturbations within microbial communities; however, it lacks the ability to factor in host-specific modulators of the gut microbiota.

In vitro Simulation of the Host-specific Gut Microenvironment

Most microbiota studies have been carried out on animal models; however, this method is costly, both from financial and temporal standpoints. A review outlining the study of the microbiota in animal models has discussed the importance of animal models in microbiome studies [69]. While animal models hold great importance for understanding host-microbe interactions, advancements in engineering of biomaterials have recently provided an optional approach to study the complex interactions occurring within the gut microbiome. It is now possible to construct *in vitro* systems that simulate intra-organ micro ecosystems, permitting high-throughput microbiome studies by engineering microfluidic devices that can be seeded with cultured host cells [70]. The system provides a method for studying microbial interactions in a highly controlled 'host' background. These gut-on-a-chip systems have a number of potential uses including nutrition research (Box 2). By having precise control of nutrient input, effects of beneficial gut modulators (**prebiotics**) on microbial content and

host cell health could be assayed. Microfluidic devices can also be used to study microbe-microbe interactions, including microbial behaviors [71]. As an example of microbe-microbe interactions, chemotaxis/chemical attraction has been shown to be more effectively studied using microfluidic devices than traditional capillary-based assays, showing chemotactic velocities of over double values obtained utilizing previous methods (as high as 35% of swimming speed for *E. coli*) [72].

In another recently developed *in vitro* system, colonic and intestinal stem cells were employed to grow three-dimensional 'organ-like' structures within a matrigel overlay without utilization of microfluidics technology [73]. Using a microraft array, individual colonospheres (spherical three-dimensional structures grown from colonic stem cells) or enterospheres (intestinal stem cells) were collected from an array containing individually grown structures [74]. The three-dimensional spheres continued to grow, budding and forming crypts, simulating the gut *in vivo*. These microstructures have been primarily used to study stem cell differentiation and growth, but this technology provides a valuable platform to study gut microbial interactions. Microbial suspensions can be microinjected into the pseudo-lumen of colonoid or enteroid structures, which then could be recovered and assayed for microbial content, microbial transcriptomics, and host gene expression profiles. This technology is still in its infancy, but may provide novel and valuable methods for higher throughput microbiome studies than existing models. Moreover, addition of automated injection and harvesting mechanisms may provide in the future a platform for high throughput microbiome studies.

Advancements in Gut Microbiome Modulation and Host Health

Advancement of microbiome research technology converges at one point: the identification and study of **microbiome modulators** and their impact on host health. As technology and research advance, an iterative process is established where well characterized modulators can be applied in the discovery of other parameters that impact the gut microbiota. Any compound, microbe, or environmental factor that results in a compositional or functional modification of the microbiome can be considered a microbiome modulator. Modulators can act as short-term modulators or as long-term modulators, and can result in microbiome alterations either beneficial or detrimental to host health (Table 1). As such, modulators could be used to specifically alter microbiome function or content as a mechanism to prevent and treat diseases.

Studies have highlighted diet as the most relevant modulator of the gut microbiome [3, 75-79], which can function as both a long and short-term modulator and act as either a beneficial or detrimental modulator. High fat diets, for example, promote growth of Firmicutes and decrease Bacteroidetes, resulting in a microbial composition associated with obesity [80]. Consequently, dietary supplementation is one of the most easily performed means to beneficially modulate the microbiota [81, 82]; however, the specific activities and impact of supplements are unknown due to the complexity of the microbial community. For instance, vitamin B12 is a limited resource within the gut, and supplementation promotes growth of microorganisms that would otherwise be restricted by its absence but the precise mechanisms and pathways involved in B12 metabolism have not yet been elucidated [82].

Dietary supplementation with prebiotics also functions to positively modulate the gut microbiota by promoting growth of beneficial microbes [79, 83]. While humans are unable to metabolize specific ingested compounds, the microbes residing within the gut, predominantly the distal colon, are rich with metabolic diversity. This vast metabolic potential allows for the breakdown of indigestible compounds into components that may be utilized by the host or by other microbes [79, 84]. For example, dietary galacto-oligosaccharides (GOS) promote growth of *Bifidobacterium* and other beneficial bacteria by selective fermentation by gut microbes [85-87]. As the information regarding prebiotic modulation of the gut microbiota becomes more readily available, new prebiotic compounds will undoubtedly arise with specific modulatory functions.

Likewise, it is accepted that **probiotic**s generally have a short-term beneficial effect on the gut microbiota [88, 89], promoting growth of other beneficial microbes, and facilitating host immune response. Members of the lactic acid bacteria (LAB) group including several species of lactobacilli are commonly regarded as probiotics. One of the most commonly studied probiotic *Lactobacillus* species is *L. rhamnosus*, specifically *Lactobacillus rhamnosus* GG (LGG). LGG was isolated from a healthy human adult and identified as a probiotic bacteria based on its resistance to the conditions of the foregut and its ability to adhere to mucosal membranes surrounding gut tissue [90]. *L. rhamnosus* also has an effect on host gene expression and immune response, activating nuclear factor-κB (NF-κB), protein kinase C, and phosphoinositide 3-kinase (PI3K) pathways [91, 92]. Probiotics including species of *Bifidobacterium, Lactobacillus,* and *Saccharomyces* are regularly administered as interventions to correct dysbioses caused by disease, chemotherapy or other drug treatments [89]. By understanding the molecular mechanisms by which probiotic microbes modulate the gut microbiota through a combination of NGS and microbiology approaches, it becomes possible to develop tailored microbial interventions to treat or prevent specific chronic and acute gastrointestinal diseases.

Novel methods for selective microbiome modulation, including antibiotic treatment [93], bacteriophage therapy [94-96], dietary supplementation [87], fecal microbiota transplantation [66, 97], and probiotic microbial interventions [88] all provide opportunities to specifically control the composition and/or function of the gut microbial community (Box 3).

Concluding Remarks and Future Directions

There is a wealth of untapped information within the gut microbiome. Information about the countless biological processes occurring in this complex microbial community, which are not entirely understood, provides valuable insight into microbial impact on host health. The data obtained by high-throughput sequencing contains more information than can be analyzed effectively using existing bioinformatics platforms and microbiology techniques. However, as technology advances, it becomes easier to delineate this data, elucidating mechanisms by which microbes impact their host and other members of the gut microbial community. Identification and characterization of previously unknown modulation mechanisms of the gut microbial community will facilitate the advent of technology for microbiome editing to prevent, detect and treat disease (see Outstanding Questions).

With faster and more cost effective sequencing platforms and data analysis pipelines, the idea of using microbiome content as a biomarker for disease is rapidly becoming a possibility. Furthermore, advancements in culturing techniques allow for the customization of synthetic microbial consortia and permit the study of microbe-microbe interactions within complex systems. Therefore, the ability to customize gut microbial consortia will advance individualized medicine. By using high-throughput cultivation technology of a previously characterized microbial community, the gut microbiota of an individual can be grown and used to derive modulators that will specifically repair dysbioses within that individual, potentially reducing or curing disease.

Together, these technological advancements make new gut microbiome research initiatives possible. As a major modulator of human health, the gut microbiome will continue to attract researchers from diverse scientific backgrounds, while advancements in technology from a wide range of scientific fields will continue to provide the tools needed to further unlock the potential of the gut microbiota as a target for personalized medicine.

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Glossary

16S rRNA
Universally conserved bacterial ribosomal RNA gene. 16S rDNA is regularly the target for sequencing microbial metagenomes.

Cultivable
Able to be grown conventionally by traditional microbiology approaches.

Dysbiosis
Compositional or functional shift within host-associated microbial communities that have the potential to facilitate growth of pathogens and/or onset chronic diseases.

Denaturing gradient gel electrophoresis (DGGE)
Method that utilizes a poly-acrylamide gel with increasing concentration of denaturing chemicals (SDS, urea, etc.) at constant temperature. PCR products run through the gel separate based on nucleotide composition, and different sequences appear as different bands on the gel

Metabolome
Profile of existing intermediate and end products of metabolic pathways within a system under a given set of conditions.

Microbiome
All nucleic acids composing a complex microbial community.

Microbiome modulators

Any compound, microbe, or environmental factor that results in a compositional or functional modification of the microbiome.

Microbiota
Ecological community of commensal, symbiotic and pathogenic microorganisms in a particular site, habitat, or geological period

Microfluidics
Multidisciplinary field intersecting engineering, physics, chemistry and biology. Microfluidics systems are designed such that low volumes of fluids can be processed to achieve high-throughput screening, multiplexing, and/or automation.

Prebiotic
A selectively fermented compound that results in specific changes in the composition and/or activity of the gastrointestinal (GI) microbiota thus conferring benefit(s) upon host health.

Probiotics
Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.

Temperature gradient gel electrophoresis (TGGE)
Similar to DGGE but uses variable temperature and a constant detergent concentration.

Terminal-restriction fragment length polymorphism (T-RFLP)
PCR amplification with labeled primers generally targeting the 16S rRNA gene followed by restriction digest of products and detection of fluorescent-labeled fragments. Since different sequences yield different sized fragments, profiles of microbial communities based on sequence dissimilarities can be generated through this procedure.

Metatranscriptome
All RNA molecules, including mRNA, rRNA, tRNA and other non-coding RNA transcribed in a population.

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Box 1. Bioinformatics Tools Applied to Gut Microbiome Research

Microbiome bioinformatics seeks to provide computational methods and techniques, complementing experimental approaches, to enrich our understanding of complex microbial communities, their internal interactions, and their interactions with host and environment. Current bioinformatic practice, in many respects encapsulated in the software platforms Mothur [98] and QIIME [29], focuses on processing high throughput sequencing of variable regions of bacterial 16S ribosomal genes with an emphasis on exploratory data analysis and visualization of taxonomic composition. Emerging bioinformatics approaches enhance these techniques with improved statistical rigor and provide insight into the complex environmental context through complementary taxonomic diversity analysis, whole genome shotgun (WGS) and metatranscriptomic sequencing.

At its most fundamental level microbiome bioinformatics is founded on the concept of operational taxonomic unit (OTU) determination. Although relatively naive hierarchical clustering models can be sufficient in some analyses, a much richer probabilistic model of clustering provides a much more nuanced analysis that is relatively free of the potentially negative ramifications of having to select arbitrary thresholds for parameters that are difficult or impossible to estimate given experimental data. Recent developments in OTU selection focus on vitiating the effects of essentially arbitrary parameters [99], determining what complementary information can be gleaned from the sequencing results without OTU selection [100, 101], and deriving the most information from the OTUs that are selected. Oligotyping [102, 103] applies information theoretic approaches to subsets of reads identified *a priori,* such as those reads within a particular OTU or categorized within a particular phylum, to differentiate biologically relevant distinctions resulting in significantly improved taxonomic resolution. Bayesian approaches to OTU selection, such as MicrobeDMM [104], are proving successful in a number of studies [104, 105]. These approaches not only afford a richer probabilistic model of clustering, but also inherently support the inclusion of prior information and additional information sources.

As data sets from diverse experimental sources become available, the possibility of exploiting complementary bioinformatics analyses becomes possible. In addition to providing robustness to spurious conclusions and enhanced reproducibility, statistically rigorous methods provide meaningful metrics to evaluate and compare bioinformatic results across diverse analyses required for comprehensive meta-analyses. In order to achieve a meaningful synergy of diverse information sources such as taxonomic [106] and functional [107, 108] profiling, the results of multiple bioinformatic analyses and meta-analyses need to be combined in a principled manner. Preliminary investigations in this direction have produced promising results [45, 109, 110], but significant development opportunities remain.

Box 2. Stem Cell Derived Organoids and Microbiome Studies

Medical research and regenerative medicine studies using stem cell technology have recently provided insight into organ development *in vivo*. Individual intestinal stem cells have been grown into fully differentiated 'mini-guts', providing a platform to study cell signaling in gastrointestinal development [111]. The field of stem cell biology has advanced significantly in recent years, allowing for the advent of high throughput 'organ-on-a-chip' systems to study development, differentiation and physiology of developing organs [112]. Gut-on-a-chip models initially utilized Caco-2 cells (a cell line derived from human colorectal adenocarcinoma [113]) grown on extracellular matrix (ECM) coated flexible membranes attached to microfluidics channels, subject to peristaltic-like movements and fluid flow [112]. This method simulated the gut microenvironment, stimulating Caco-2 cells to form three-dimensional villi-like structures [114]. The micro-devices were predominantly used to study physiology of host cells under various treatment conditions, including co-culture with specific microorganisms. Use of this three-dimensional microenvironment colonized with gut dwelling microbes [70] demonstrated that microbe-induced inflammation within epithelial cells was sufficient to compromise the intestinal barrier [115].

Stem cells are capable of initiating morphogenesis generating complex structures *in vitro.* Consequently, use of stem cell-derived organoids colonized with microbial consortia can allow for a much more detailed study of microbe-microbe interactions in a highly controlled microenvironment providing a high throughput alternative to gnotobiotic live animal models. Membrane-free three-dimensional stem cell derived organoids have been generated using individual intestinal stem cells or intact crypts harvested from mice [74] and human induced pluripotent stem cells [116]. These organoids, which contain various differentiated cell types, form a barrier similar to that of intestinal or colonic epithelia [74, 116] and have been recently used to show that *Salmonella enterica* serovar Typhimurium can successfully invade the epithelial cell layer [116] and that *Clostridium difficile* can disrupt epithelial barrier function [117]. These studies opened the door for the use of stem cell technology to study interactions within the gut microbiota, as well as between the microbiota and its host.

Moreover, the genetic background of the stem cell derived organoids can be controlled; as stem cells harvested from a living subject can be grown into organoids (Figure I). This permits for host-specific microbiota-directed disease interventions to be screened for, and tested in a high throughput fashion prior to live animal testing. This may ultimately allow for the identification and testing of patient-specific interventions for chronic gastrointestinal diseases much more rapidly than has ever been possible in the past.

Box 3. Selective Modulation of the Gut Microbiota: Microbiome Engineering

Gut dysbioses have been repeatedly linked to human disease; hence the gut microbiota has become an increasingly attractive target for disease interventions [2, 118, 119]. By using modulators of the gut microbiota, composition and function of the microbial community can be selectively altered. Mechanistic studies of gut microbiota modulation are scarce [79], however, knowledge from studies of that nature will allow state-of-the-art modulators to arise as treatments for chronic and acute human diseases.

Amongst recognized selective modulators of the gut microbiota are bacteriophages, probiotics, prebiotics, antibiotics, and engineered synthetic bacterial consortia. Bacteriophage-mediated culling of specific members of the microbiota may alter microbial composition and free previously occupied niches for novel colonization [95]. Conversely, dietary prebiotic supplementation promotes growth of beneficial microbes, allowing them to survive and colonize their host better than others within the community[63, 87, 120]. Probiotic modulation often combines these two mechanisms, both promoting growth of beneficial microbes and selecting against microbes that may be detrimental to the host [88, 90]. Similar to probiotics, use of fecal microbiota transplants from healthy donors, or cultivated by *in vitro* gastrointestinal simulators have recently arisen as a method to restore gut dysbiosis as well as to treat gastrointestinal disease by almost instantaneously modifying microbial composition and the gut microenvironment [97, 121].

Combinatory modulation of the gut microbiota is an emerging frontier in gastrointestinal medicine. Antibiotic treatments have been shown to enhance the efficacy of microbiota transplants, improving the likelihood of colonization by implanted microbes [122]. Prebiotic treatments in conjunction with probiotic supplementation allow for more effective growth of probiotic microbes and improved function[63].

Finally, microbiome engineering and synthetic biology will demonstrate mechanisms by which it may become possible to selectively engineer the composition of the gut microbiota. Engineered T7 bacteriophages containing modified tail fibers have already been generated with the ability to infect and eradicate specific microbes from a population based on tail fiber specificity [96]. This modular construction of synthetic bacteriophages allows for very specific targeting of a very broad array of different bacteria. Moreover, novel delivery systems permit bacteriophages to be administered as food supplements, improving localization and impact of the treatment [123]. Combining these technologies may provide a method to selectively eliminate individual detrimental members of a microbiota as a way to fight dysbiosis and disease.

Outstanding Questions

-Can the gut microbiota be used as a reliable biomarker for early detection of chronic diseases? Advancements in NGS technology allow for rapid identification of members of microbial communities. If dysbiosis within the gut can become a reliable biomarker for chronic disease, it may be possible to devise strategies to treat these diseases prior to onset.

-What are the impacts of uncultivable microbes within the gut on their community and host? Mechanistic studies of microbes are dependent on microbial cultivation. Advancements in cultivation technologies will provide information regarding network interactions between host, community, and previously uncultivable microbes.

-What role do bacteriophages play in the gut microbiome? As understudied members of the gut microbiome, the diversity and role of bacteriophages within the community is often overlooked, and in many cases unknown.

-How do various members of the gut microbiota modulate host gene expression? Can this information be useful in disease prevention? Gut microbes influence host gene expression directly and indirectly. While not all microbial mechanisms to modulate host gene expression are known, more and more information regarding microbiota-modulated host gene expression is emerging as technology advances. Knowledge of how host gene expression is controlled by microbes may allow for the engineering of microbes to selectively control expression of target genes.

-Will the use of patient stem cell derived organoids and microbiota become common practice in the development of personalized treatments for chronic gastrointestinal diseases? By using freshly harvested intestinal stem cells, organoids can be grown containing the precise genetic background of the patient. These organoids can subsequently be colonized by the microbiota from the patient, and subjected to treatments in a high throughput fashion in order to test efficacy of specific interventions.

Trends Box

-Compositional and functional analyses of the gut microbiome by next-generation sequencing methods have completely transformed research approaches to determine microbial species and their role in human health and disease.

-New culturing technologies facilitate mechanistic studies of difficult to culture gut microbes. A number of previously uncultivable microbes are now cultivable as a result of these advancements, providing insight into community dynamics and network interactions.

-Stem cell technologies and tissue engineering allow for construction of organoids capable of facilitating cost effective, high-throughput microbiome studies.

-Modulation of the gut microbiota is emerging as an effective method to delineate composition and function of microbial communities providing new methods to prevent and treat disease.

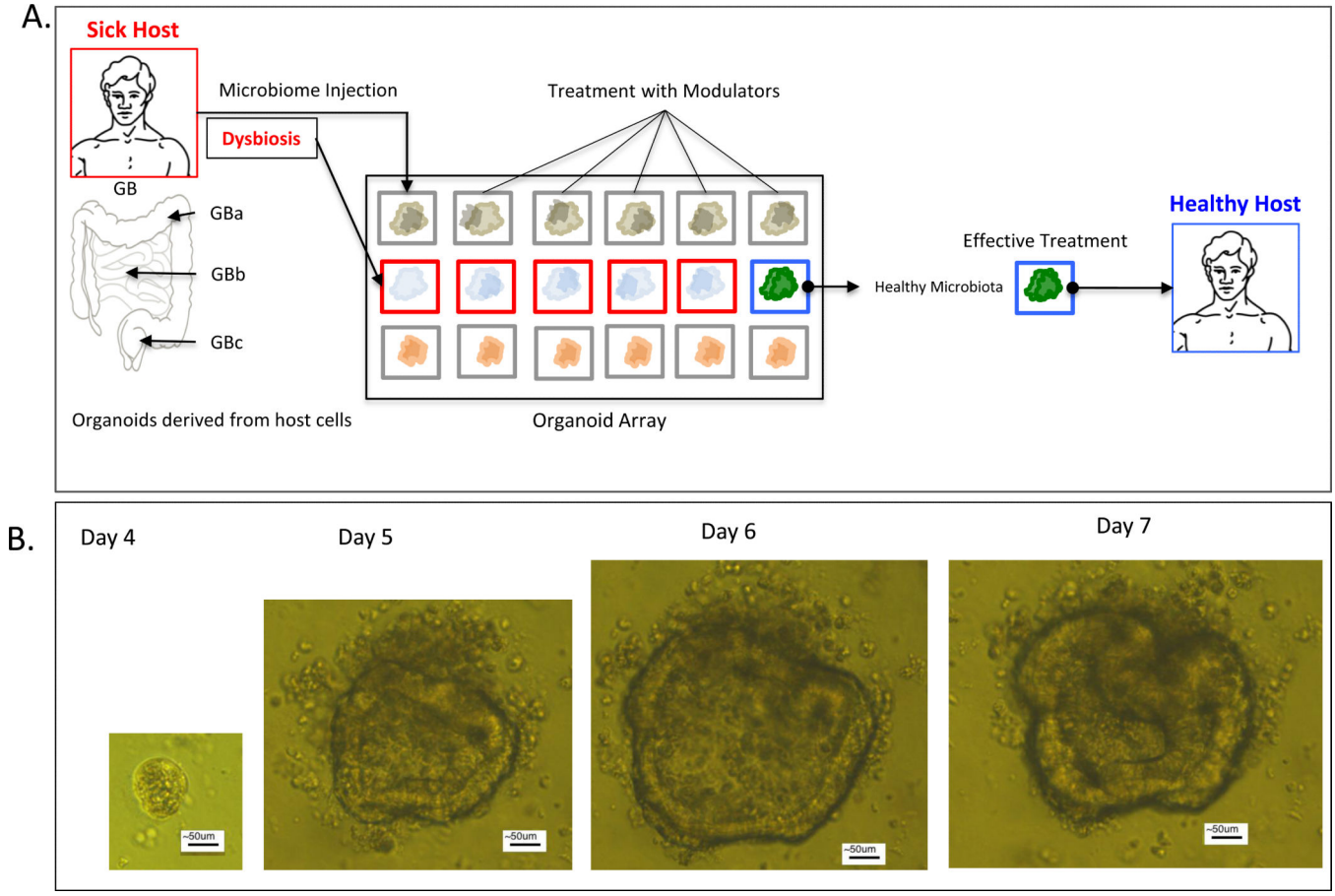

Figure I. A Microbiome-Organoid Array to Study the Impact of Gut Microbiome Modulators on Individual Hosts

A) Host-derived organoid arrays could derive personalized treatments and diagnostics based on genetic background (GB) and gut microbiome composition. Individual organoids can subsequently be harvested and screened for microbial composition, microbial transcriptome, and host gene expression with the aim of identifying an effective treatment for the individual. B) Mouse small intestine stem cell-derived enteroid from days 4 to 7. Photographs show progression of the organoid from a sphere to mature colonoids presenting crypt-like structures.

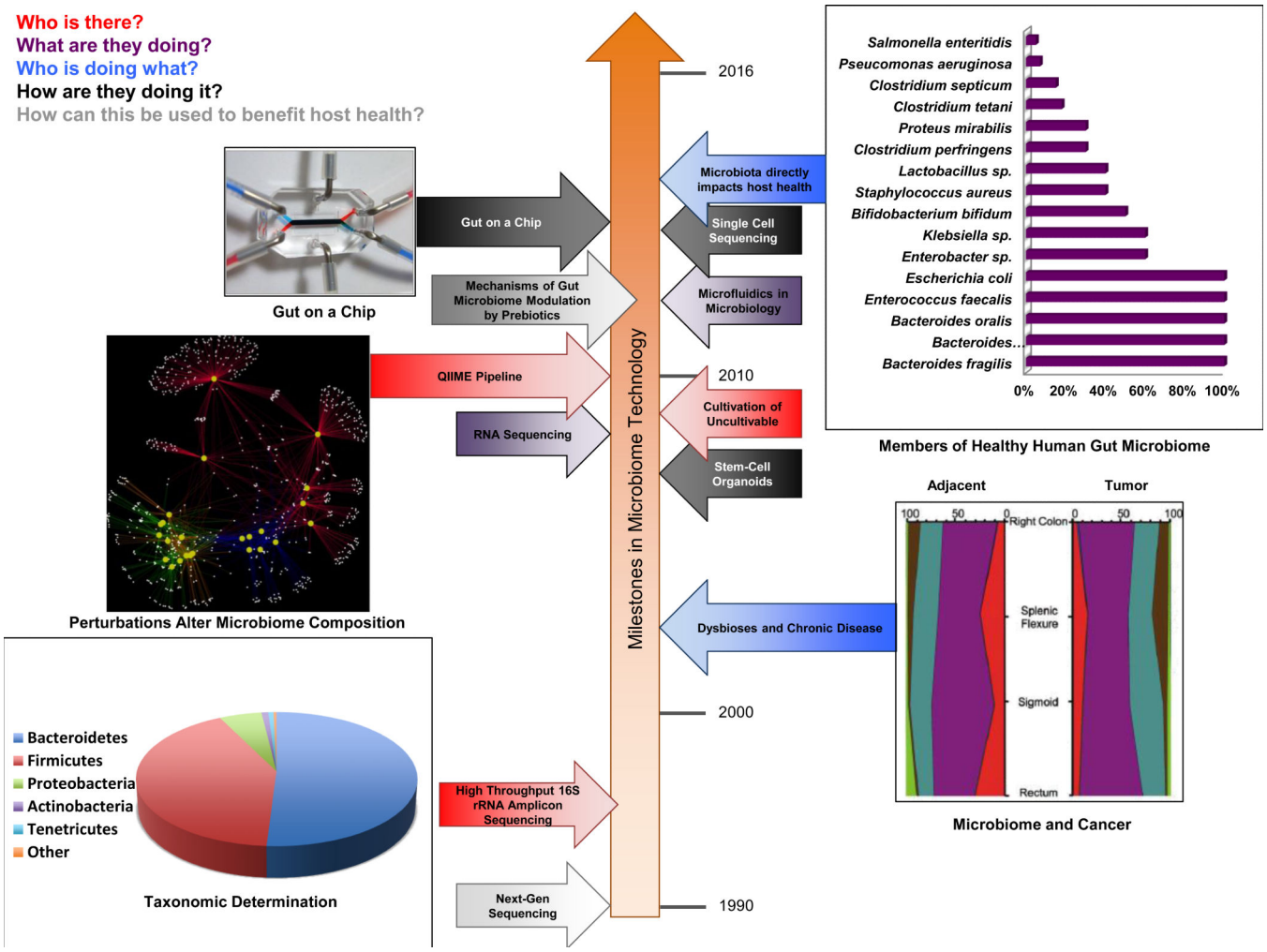

Figure 1.

Timeline of major advancements on gut microbiota studies. Research centered around low-cost high-throughput next generation sequencing began to emerge in the past 10 years. These advancements have provided the opportunity to study complex communities and their impact on host health, and culmination of these technologies is driving the field toward a future of personalized medicine. Taxonomic determination of microbes dwelling in the gut by 16S rRNA sequencing was first performed in the late 1990s. Dysbiosis and its impact on human disease was first discussed in early 2000s, and identified in colorectal cancer [133] among other diseases including Crohn's disease. Since 2010, rapid advances in bioinformatics including Quantitave Insights into Microbial Ecology (QIIME) for analysis of metagenome have been used to identify core members of the gut microbiota of healthy humans [134] as well as the impact of perturbations on gut microbial communities [135]. Finally, biology and engineering sciences converged in the design of ‘Gut on a Chip’ devices to simulate gastrointestinal environments [70].

Table 1

Examples of Modulators of the Gut Microbiota

Modulator type	Example	Impact on host	Duration/rate	Effects on the microbiota
Prebiotics	β, 1-4 Galacto-oligosaccharide (GOS)	Beneficial	Short-term/intermediate	Promotes growth of *Bifidobacterium* and *Lactobacillus* [124] Inhibits growth of *Clostridium* [125] Increases recovery rate of microbiota post-antibiotic treatment [126]
Probiotics	*L. rhamnosus GG*	Beneficial	Short-term/intermediate	Inhibits growth/colonization of pathogenic microbes [90, 127] Promotes growth of *Bifidiobacterium sp.* [128]. Modulates host gene expression [90]
Bacteriophages	933W *coliphage*	Detrimental	Short-term/rapid	Modify and/or eradicate populations of commensal *E. coli* [129]. Transmits endotoxin genes to bacteria within community [130].
Antibiotics/drugs	Chemotherapy	Beneficial or detrimental	Short-term/rapid	Culling of microbes to free niche space [122]
Host immune response	Toll-like receptor mediated gene expression	Generally beneficial	Long-term/rapid	Inhibits colonization of certain microbes. Eliminates invading pathogens from the population. [46]
Diet	High fat vs. low fat diets	Beneficial or detrimental	Long-term/slow	Vitamin supplements impact transcription and microbial content [82, 131] High fat diets promote ‘unhealthy’ microbiota [80]
Transplantation	Fecal Transplant	Mostly beneficial	Long-term/immediate	Transplantation of healthy microbiota can eliminate *Clostridium difficile* infection [97]
Pathogenic bacteria	*Salmonella sp.*	Detrimental	Short-term/intermediate	Outcompetes other microbes within the microbial community, reducing community diversity [132].