Title: The new microbiology: Cultivating the future of microbiome-directed medicine

Running title: The new microbiology

[1]Connie W.Y. Ha and [1]Suzanne Devkota*

[1]Division of Gastroenterology, F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048

***Corresponding author:**

Suzanne Devkota PhD
Cedars-Sinai Medical Center
Davis Bldg 4011
110 George Burns Rd.
Los Angeles, CA 90048
Email: Suzanne.Devkota@cshs.org

Abstract

The discovery of human-associated microscopic life forms has captivated the scientific community since their first documentation in the 17th century. Subsequent isolation and cultivation of microorganisms have spurred great leaps in medicine, including the discovery of antibiotics, identifying pathogens that cause infectious diseases, and vaccine development. The realization that there is a vast discrepancy between the number of microscopic cell counts and how many could thrive in the laboratory motivated the advent of sequencing-based approaches to characterize the uncultured fraction of the microbiota, leading to an unprecedented view into their composition and putative function on all bodily surfaces. It soon became apparent that specific members of the microbiota can be our commensal partners with new implications on various aspects of health, as well as a rich source of therapeutic compounds and tools for biotechnology. Harnessing the immense repertoire of microbial properties, however, inadvertently requires pure cultures for validation and manipulation of candidate genes, proteins or metabolic pathways, just as mammalian cell culture has become an indispensable tool for mechanistic understanding of host biology. Yet, this renewed interest in growing microorganisms, individually or as a consortium, is stalled by the laborious nature of conventional cultivation methods. Addressing this unmet need through implementation of improved media design and new cultivation techniques is arguably instrumental to future milestones in translational microbiome research.

Introduction

For more than one century scientists have largely relied on cultivation approaches to establish the physiological and biochemical properties of microorganisms. These efforts not only facilitated the discovery of anaerobic life forms that colonize mammalian systems, the means to propagate organisms on solid culture media paved way for the Golden Age of Microbiology. As of 2020, the total number of valid published names of bacteria in the DSMZ database exceeded 19,000 type strains that span over 3,000 genera (50). The ATCC Mycology Collection has accumulated more than 7,600 species of fungi and yeasts, 300 of which are biomedically-relevant (38). While this appears to be a substantial assemblage of cultivable microorganisms, it is estimated that there are as many as 10^{12} species of bacteria, archaea and fungi exist on Earth (48). In fact, 55 out of 92 distinct bacterial phyla have no cultivable representatives (24) Reasons why only a fraction of all viable organisms from a given specimen can be isolated and grown in lab conditions include: i) dormancy of microbial growth once they were removed from the natural habitat (14); ii) lack of highly specific but essential nutrient requirements or signalling molecules (9) and; iii) complex cross-feeding relationships between members of the microbial community (44). Thus, exploration of the diversity and function of our microbial selves have been progressively replaced by culture-

independent approaches, ranging from Sanger sequencing, high-throughput sequencing of 16S amplicons, shotgun metagenomics, meta-transcriptomics to single-cell genomics.

The sequencing revolution has facilitated characterization of the uncultured organisms that make up the human microbiota and the genes they carry. A sequencing-based survey by the initial Human Microbiome Project has revealed that the total human microbiome contained between 3,500 and 35,000 species-level taxa, spanning roughly 600 genera (15). More importantly, culture-independent studies brought novel insights linking human health and commensal organisms. Diseases like obesity, allergies, asthma, and inflammatory bowel diseases are associated with the presence or absence of certain microbial groups and influenced by the age at which we are exposed to them (31, 54-56, 69). Distribution of microbial constituents and their genes are not only linked to disease status, they are profoundly shaped by several factors, including lifestyle, age, body site and diet history of the studied individuals (5, 16, 39, 53, 66). Currently, sequencing technologies have advanced to the point where some sequencing platforms are pocket-sized, with the capability to draw power from and transmit data to a laptop through a USB connection (58). This opens new avenues for potential applications in public health surveillance in remote settings, or even monitoring microbial dynamics in microgravity (43, 57).

Without a doubt, culture-independent exploration of the microbiota has led to fundamental biological discoveries. We now have an expansive directory of human-associated microorganisms and an appreciation of their putative functions based on reconstruction and annotation of microbial genomes, but that is not sufficient to fully harness and/or mechanistically tease out complex microbe-microbe or host-microbe interactions. In this review, we first discuss why culture-based studies are still relevant in modern microbiology and microbiome sciences, and secondly, explore the ways in which cultivation can be improved and applied to future studies of the human microbiota.

Why do we need cultivation?

Renewed interest in cultivation has been motivated by the realization that there are limitations to profiling and cataloguing the microbiome via sequencing. Microbial ecosystems typically have an uneven abundance distribution, in which a handful of dominant organisms would co-exist with relatively high number of rare organisms (46). Low abundance, but biologically relevant populations may be excluded from sequencing-based analysis for a range of technical reasons. DNA extraction protocols differ in cell lysis efficiency (40), and that may have a major impact on the recovery of genomic content from difficult-to-lyse organisms within the specimen. As noted by Fiedorová et al, finding one extraction protocol capable of sufficiently lysing gram positive bacteria and fungi in hyphae, yeast or spore forms is challenging as the cell wall of these microbial forms are more resistant to degradation (19). The success of amplicon-based sequencing is highly

dependent on the choice of primers. Primer pairs targeting different regions of the 16S rRNA gene can generate different community profiles, leading to inaccurate assessment of bacterial diversity of the sampled site (12). Sequencing depth is another key determinant in identifying the constituents that make up the human microbiota (7). For instance, if there are 10^{14} microorganisms in the intestines, a 16S rRNA amplicon sequencing run that generates 500,000 reads would capture five bacterial cells in every billion with the assumption each cell carries one copy of the 16S gene (23). The detection threshold for amplicon-based sequencing of frequently used sequencing methods is estimated to be 10^6 microbial cells per gram of stool (35). Patterns reported in such studies are likely limited to signatures of high or moderately abundant organisms linked to a particular physiological state. Yet, rare organisms can serve as the keystone species in regulating the functioning of host-associated environment (27). In contrast, culture-based methods can achieve high level of sensitivity via rounds of *in vitro* selection and enrichment to enable the capture and characterization of the minority microbial populations (45). Even bacteria present at $<10^3$ cells per gram of stool can be detected by cultivation (35).

Aside from bridging the gap in detection bias, cultivation efforts can also inform taxonomic and functional assignments of short sequence reads from metagenomic, meta-transcriptomic and meta-proteomic studies. A critical step in extrapolating community-, species- or strain-level information from the entire genomic content of any human-associated microbe is the alignment of sequences to reference databases, such that genes that co-vary with environmental or host traits can be precisely binned and annotated into functional or taxonomic clusters. However, of all the bacterial genomes deposited in the NCBI database less than 4% belong to commensals of the human gut because historically the focus of many genomics studies was on pathogenic, antibiotic-resistant bacteria, which are over-represented across multiple microbial databases (71). Thus, it is not surprising that more than half of the reads from a typical human gut metagenome cannot be mapped to existing bacterial reference genomes (68). The roadblock in data mining can be attributed to the absence of well curated, high-quality bacterial reference genomes. Finer-scale analyses of the microbiome, including, SNPs and strain variations, rely heavily on the coverage and quality of reference genomes. Bacterial genomes can be generated from *de novo* metagenomic assembly of a mixed microbial community, but they are more likely to be incomplete or may represent chimeric species populations in comparison to those generated from pure cultures (51). Hence, isolating and growing individual organisms for whole genome sequencing is still invaluable for expanding existing databases, and this resource will make it much easier for researchers to determine which organisms are present within a population and interrogate their role in disease.

Datasets from extensive high-throughput sequencing efforts and corresponding metadata have unravelled a plethora of host-microbe associations related to metabolic interactions, disease

severity, immune modulation and therapy success. But to help determine cause and effect, and to narrow down on constituents of the microbiome that mechanistically link to a specific host trait, cultivation of individual strains or a well-defined consortium for further *in vitro* or *in vivo* experiments in animal models is required. By co-culturing a synthetic consortium of 14 putative fiber-degrading bacteria in a bioreactor spiked with prebiotic inulin, and removing one species at a time, Gutiérrez and Garrido were able to tease out complex cross-feeding patterns and identify which species are key for the production of diet-derived metabolites that have immunomodulatory properties with health implications (21). Without the means to isolate and reliably culture commensals from the gut, it would be almost impossible to validate that a cocktail of 17 Clostridial strains, or supplementation of their short-chain fatty acid byproducts, can induce regulatory T cell responses and attenuate disease in models of colitis and allergic diarrhea, as shown by Atarashi et al. (3). This study, among many others, draws attention to the fact that organisms that live in and on us are an underexplored resource of probiotics or natural products for therapeutic purposes. Pure cultures can also help map out complex diet-host-microbe interactions. Culture-based assays have shown that specific strains of *Eggerthella lenta* are able to inactivate the cardiac drug digoxin (63), and that dietary arginine can reduce microbial metabolism of digoxin *in vivo*, with consequences in drug bioavailability (22). Implications of such cultivation-based findings suggest that personalized dietary guidelines coupled with an assessment of patients' microbiomes may be useful for guiding medication regimes. These studies demonstrate that an extensive culture collection offers flexibility in experimental validation of sequence-based predictions, and ultimately, opens new therapeutic options.

Why microbial cultivation is a dying art

As high-throughput meta-omics approaches become indispensable for exploring the composition and functional properties of microbial ecosystems, culture-based methods are often perceived as an old-fashioned technique that has fallen by the wayside. This was perhaps perpetuated by "the great plate count anomaly" which showed that only a fraction of microorganisms observed microscopically could be propagated and identified on a petri dish (67), however the popular belief that only 1% of microorganisms can thrive in the laboratory settings is not entirely true. First, it must be noted that the 1% number refers to the totality of bacteria on earth, not the human body. When referring to mammalian systems, many are in fact culturable, especially intestinal microorganisms of human and mouse origin. Lagkouvardos et al., determined that 35-65% of species detected by sequencing have representative strains in culture (37), but growing them in anaerobic chambers is space and time-prohibitive for many researchers. To put things into perspective, Lagier et al., required more than 70 different culture conditions to identify 340 species of bacteria in human stool (35). The authors determined, however, that the number of

culture conditions does not necessarily scale linearly with the number of unique taxa. In fact, 73% of the identified species could be recovered by 20 culture conditions (35). Nonetheless, isolating all the unique colonies on this subset of conditions for pure culture is undeniably an elaborative process. The study by Lagier et al., illustrates that the growth of many organisms cannot be supported by a single artificial media, instead requiring various optimized media containing essential growth substrates present in the microbe's native environment which can be difficult to source or formulate. Soon after primary isolation and cultivation, organisms of interest should be appropriately archived, e.g. storage in cryoprotectant below -70°C. The number of passages from the original culture should be minimized to reduce the possibility of phenotypic variations and genetic drift (16), as microbial adaptation to artificial culture media may skew the interpretation of downstream *in vivo* or *in vitro* characterization. When dealing with multiple organisms with different growth rates and nutrient requirements, setting up frozen cultures for long term storage and timely management of stock and working cultures for ongoing experiments can be challenging. In many ways microbial cultivation is an art and a craft, in stark contrast to genomic-based tools with defined protocols and code.

Another bottleneck, and often costly step in culture-based studies from mixed communities, is the identification and classification of organisms among the numerous cultures produced. Traditionally, this process involves PCR amplification and sequencing of the 16S rRNA gene of individual bacterial colony, followed by assigning the sequence to a species based on known references. In routine clinical microbiology laboratories, this approach has been slowly replaced by MALDI-TOF mass spectrometry (2), which profiles bacterial proteins from whole cell extracts and the resultant fingerprint would then be matched against a reference database for identification. This method has proven to produce fewer incorrect identifications and has a quicker turnaround time than 16S-based sequencing (70). Lagier et al. were the first to implement this mass spectrometry approach to the study of human gut microbiota, and accomplished the classification of 901,364 colonies from 1,057 bacterial species (36). Despite the laborious nature of isolating and identification of bacteria, independent investigators have discovered hundreds of novel human-associated organisms through culture-based methods (8, 20, 36), and more importantly, a portion of these organisms' identities and genes cannot be fully resolved by sequencing the stool sample directly. Together, these studies highlight how culturing can complement high-throughput sequencing, and that the combination of both culture-dependent and culture-independent approaches may allow investigators to have a more comprehensive understanding of the organisms present in a given context.

What is needed to advance cultivation?

Modernizing cultivation is the key to encouraging researchers to incorporate this methodology as part of the pipeline for routine microbiome analysis. Not all laboratories have the means to generate more than 70 growth conditions, and this calls for better culture media design to streamline the recovery of organisms from the gut or other bodily surfaces. Enriching commercially available media with animal-derived products such as sheep blood and rumen fluid, which mimics the complex nutrient environment of the intestines, has shown to be critical for isolating new species and the overall success of large-scale cultivation studies of the gut microbiota (36). However, fresh rumen fluid, for example, used in media preparation is typically sourced from fistulated cows that require special maintenance, and has limited shelf life. Although promising data suggested that freeze-dried rumen fluid is nearly as effective as fresh, while remaining stable at room temperature for months (17), commercial production and distribution of this valuable supplement are lacking. Dedicated research and development of media supplements are needed to meet the increasing demand for culturing organisms from a wide range of specimens. The availability of sequenced genomes now provides opportunities to formulate customized culture media for isolation of fastidious and/or host-dependent organisms. Investigators have begun to incorporate genomic analysis and metabolic modelling to predict the nutrient requirements of targeted organisms (4, 49, 61). In the absence of sequenced genomes, computational mining of the existing culture media catalogue and respective organisms that thrive in each media can reveal substrate preferences across the tree of life and nuances that differentiate closely related species (48). This approach enabled the development of an algorithm that predicts media formulation given an organism's 16S rRNA sequence, and more importantly, this web-based resource is now available to the public (48). Future cultivation efforts can leverage this media recommendation platform to guide media development for isolating highly sought after but difficult-to-culture organisms.

As the focus of human microbiome research shifts from association to establishing causation and molecular mechanisms, acquiring the candidate organisms identified by sequencing-based approaches will be a critical step for mechanistic inquiry. Recovering a single organism within the mixed community is akin to finding a needle in a haystack. The odds are stacked against the species of interest, especially if they are a slow-growing, minority member of the microbiota. Having the right growth media is not sufficient to guarantee cultivation success in this instance. To prevent the bloom of undesirable organisms that might compete for the same nutrients within the culture media, unconventional cultivation strategies have been implemented in recent studies to boost the selection of distinct organisms. Bacteriophages with potent bactericidal activity can be added to culture media instead of narrow-spectrum antibiotics for targeted removal of problematic fast-growers. Many phages are only effective against a specific bacterial host, therefore their presence has less interference with the recovery of other members in a microbial community than

supplementing the media with antibiotics (13). Others have used ethanol pre-treatment of human stool prior to cultivation, which has shown to be highly effective in recovering spore-forming bacteria instead of vegetative cells that may dominate a primary specimen (8), and the success of this approach highlights the importance of sample preparation in certain research contexts. Other creative approaches include the use of antioxidants such as ascorbic acid or glutathione for quenching oxygen and facilitating growth of anaerobic organisms in aerobic environments (34). Addition of these supplements in the sampling process or transport media may help promote the revival of oxygen-sensitive species in the laboratory. The examples described above have combined pre-existing culture media with new strategies to isolate targeted organisms. Innovations are not only needed in media design, creative ways to preserve, enrich or deplete specific populations are equally important to advance culture-based studies.

On the technical front, space constraints and low-throughput are the biggest hurdles in cultivation efforts. Petri dishes, flasks and culture tubes have been the workhorse for microbial cultivation in the past hundred years, but these traditional platforms are not conducive for extensive characterization of specimens that are densely colonized by thousands of unique species. Currently, there are a number of systems being developed that have the potential to bypass the cumbersome nature of traditional vessels. Advances in microfluidics have made it possible to encapsulate individual organism and culture media into microdroplets or gel particles (28, 29, 42). These approaches partition single-cells into miniaturized growth chambers and create a higher-throughput cultivation platform. This concept of compartmentalization also addresses the issue of competition and antagonism among the mixtures of organisms cultured together in the media and gives otherwise difficult-to-grow species the opportunity to expand to larger densities and increase the probability of subsequent detection. Although these automated platforms were intended for drug discovery and identification of organisms in environmental samples, the technology can be applied to human specimens to achieve similar goals. Given the footprint of these encapsulation systems, they are more suited for characterizing aerobic or aerotolerant organisms due to limited bench space within the anaerobic chamber. To address this issue, array-based miniaturized diffusion chambers with hundreds or thousands of microwells are in development which may be more appropriate for handling samples dominated by anaerobic species. The ‘isolation chip’ and ‘micro-Petri dish’ are examples of highly portable micro-culture chips that contain ultra-small compartments for isolating organisms within a mixture while allowing for passage of nutrients supportive of their growth (6, 25, 47). By adjusting the dilution of specimen, these chips can capture an individual bacterial cell into each compartment and encourage clonal expansion. A variant of the isolation chip has shown success in isolating novel anaerobic species of the human oral microbiome (65), suggesting micro-well arrays are a promising tool for cultivating organisms from human-derived specimens. Microfluidic organ-on-a-

chip systems are another platform for keeping oxygen-sensitive organisms alive. The latest iteration of the gut-on-a-chip from Jalili-Firoozinezhad et al. allows precise control and measurement of the hypoxic environment within the chip, which enables the co-culture of 200 different aerobic and anaerobic organisms with human intestinal epithelium (26). These features point to the utility of chip-based platforms in the study of mixed species biofilm formation in healthy and disease states, and bypass the need for large bioreactors to mimic the physicochemical properties of mammalian gut. An added benefit of the organ-on-a-chip system is the feasibility of assessing microbial influence on any human cells that line the microfluidic device, making for an attractive platform for mechanistic interrogation of host-microbe interactions, and modelling disease development with individual strains or a consortium of candidate strains.

Ultimately, one of the major goals of establishing a culture collection is to facilitate the discovery of functions and targets within the microbiome for therapeutic applications. There is a great need for complementary tools specialized in i) rapid detection and propagation of micro-colonies and, ii) phenotypic screening of desired traits following primary isolation. A seamless cultivation pipeline that bridges innovative culture formats with rapid identification, e.g. MALDI-TOF mass spectrometry, requires an automated system that is compatible with miniaturized growth chambers, such that picking and propagating micro-colonies can be more efficient. Combining these discrete steps in microbial cultivation in an automated platform would resolve the throughput issues of culture-based work in a range of contexts. In terms of functional characterization, multiplex phenotyping is an attractive option to validate microbial function *in vitro*. The Biolog microplate is an example of a high-throughput functional assay that exploits a colorimetric reaction to measure the response of an individual bacterial or fungal strain, or microbial community, to a large and diverse range of nutrients and chemicals (64). If desired, thousands of phenotypes may be monitored simultaneously using the different plates which can be grouped as those that measure carbon utilization (30), nitrogen, phosphorous and sulfur metabolism, response to different pH conditions and pharmaceuticals (32), and biosynthesis or degradation of small molecules (1). When dealing with a mixed community, integrating high resolution ORBITRAP mass spectrometry to the pipeline may serve dual purpose. This metaproteomics approach not only can achieve species- or strain-level identification (18, 62), but it has the potential to identify the metabolically active organisms within a consortium.

Expectations for the next decade

In the past decade, rapid generation of multi-omic datasets have been instrumental in unveiling the metabolic potential of our microbial selves. The human microbiota remains an untapped resource for biomedical applications as our data collection abilities far surpass the ability to capture and validate the putative functions of microorganisms *in vitro* and *in vivo*. Advances in

microfluidic cultivation or multi-well culture chips, and high-throughput identification of isolates hold great promises in overcoming the bottlenecks of current cultivation practices. Combining these technological breakthroughs with careful considerations in sampling strategy, transportation and custom-designed media will allow culture-based experiments to be more accessible to microbiome research. Both sequencing- and cultivation-based assays have their own limitations, advantages and utilities **(Table 1)**. The current challenge is to seamlessly integrate both arms of investigation such that our understanding of microorganisms in health and disease can be translated to clinical care.

Table 1.

	Culture-based approaches		Sequencing-based approaches	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Throughput and sensitivity	Can be highly sensitive and identify low abundance organisms.(35, 45)	Low throughput. Specimens have to be processed shortly after sampling.	High throughput. Samples can be processed in big batches.	Potential bias due to DNA extraction method, primer choice and sequencing depth. (7, 12, 40)
Detection	Captures the viable and potentially metabolically active fraction of the microbiome. (59)	Organisms can only be detected if the right growth conditions are provided. Requires *a priori* information on nutrient availability in the sampled site and substrate preferences of the targeted organisms. (8, 36)	The pipeline can work without extensive *a priori* knowledge on what organisms might be present in a given specimen. (41, 52)	Both live and dead cells are included in the readout.
Reagents, consumables and equipment	Growth media and consumables are relatively inexpensive.	Specialized equipment is needed when working with oxygen sensitive organisms. Growth supplements, e.g. rumen fluid, may be difficult to access.	Commercial kits and reagents are widely available. Sequencing itself can be outsourced to core facilities or third-party service providers.	Large data requires computational power, storage and time. This can present a bottleneck. (33) Cost increases with sequencing depth.
Applications	Useful for mechanistic studies and development of therapeutics based on live organisms or microbial products. (3, 22) Findings can inform reference databases to further sequencing-based assays. (71)	Rigorous screening, testing standards and strict regulatory guidelines may be required for microbiome-based therapies. (11)	Useful for hypothesis generating studies and screening purposes. Useful for informing machine-learning and AI computational modelling. (10, 60)	Cannot differentiate causality vs. association in most cases.

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Table

Table 1. Advantages and disadvantages of sequencing- and cultivation-based techniques in human microbiome studies.

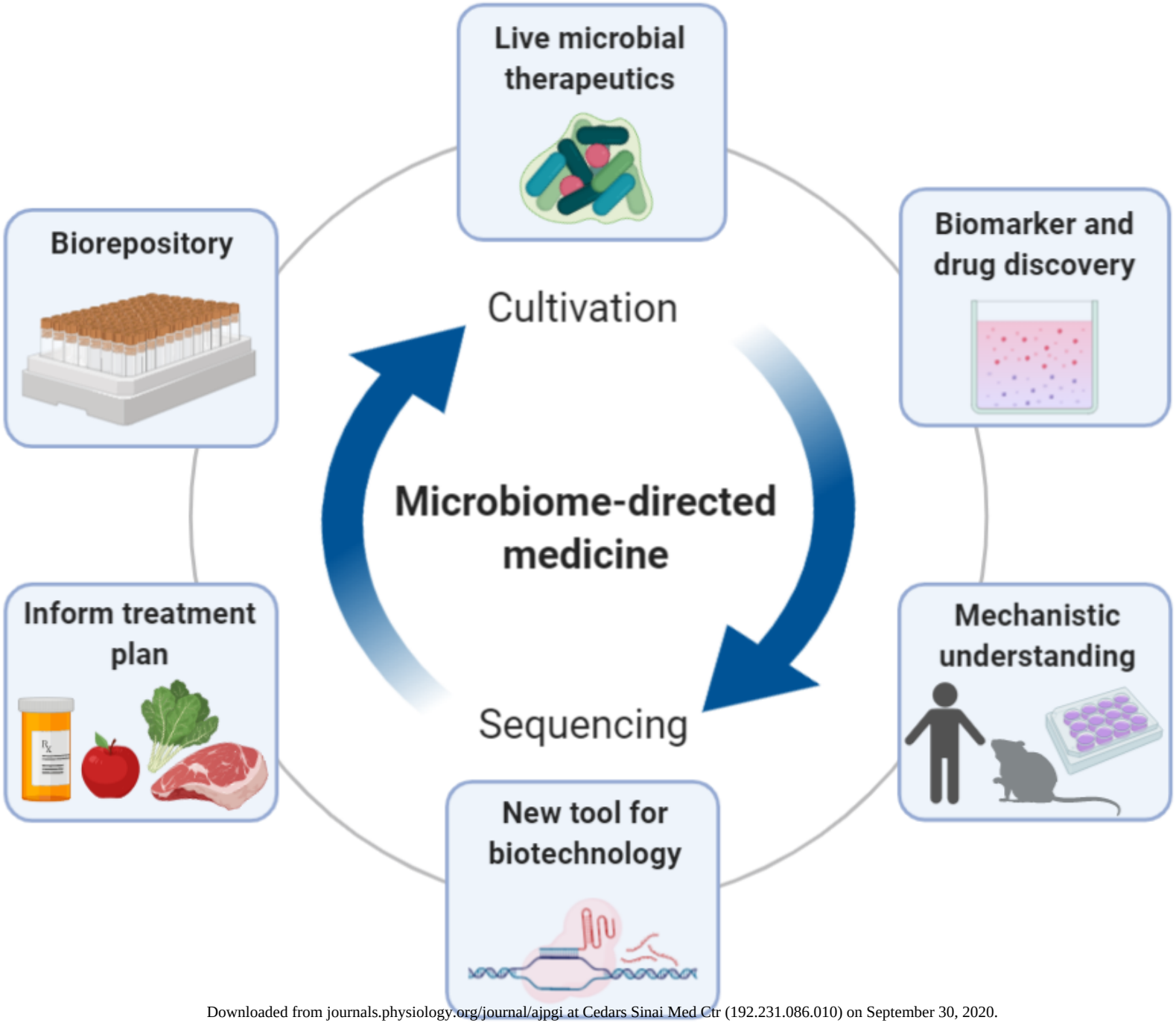

Table 1.

	Culture-based approaches		Sequencing-based approaches	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Throughput and sensitivity	Can be highly sensitive and identify low abundance organisms.(35, 45)	Low throughput. Specimens have to be processed shortly after sampling.	High throughput. Samples can be processed in big batches.	Potential bias due to DNA extraction method, primer choice and sequencing depth. (7, 12, 40)
Detection	Captures the viable and potentially metabolically active fraction of the microbiome. (59)	Organisms can only be detected if the right growth conditions are provided. Requires *a priori* information on nutrient availability in the sampled site and substrate preferences of the targeted organisms. (8, 36)	The pipeline can work without extensive *a priori* knowledge on what organisms might be present in a given specimen. (41, 52)	Both live and dead cells are included in the readout.
Reagents, consumables and equipment	Growth media and consumables are relatively inexpensive.	Specialized equipment is needed when working with oxygen sensitive organisms. Growth supplements, e.g. rumen fluid, may be difficult to access.	Commercial kits and reagents are widely available. Sequencing itself can be outsourced to core facilities or third-party service providers.	Large data requires computational power, storage and time. This can present a bottleneck. (33) Cost increases with sequencing depth.
Applications	Useful for mechanistic studies and development of therapeutics based on live organisms or microbial products. (3, 22) Findings can inform reference databases to further sequencing-based assays. (71)	Rigorous screening, testing standards and strict regulatory guidelines may be required for microbiome-based therapies. (11)	Useful for hypothesis generating studies and screening purposes. Useful for informing machine-learning and AI computational modelling. (10, 60)	Cannot differentiate causality vs. association in most cases.