Methods for defining and measuring a ‘healthy’ gut microbiome; limitations, challenges, and future prospects

Mr. Utkarsh R. Mandage[1*], Prajakta Mahajan[2], Samiksha Lokhande[3]

[1]Department of Pharmacognosy, Ravindra Vidya Prasarak Mandal Institute of Pharmacy,
Dwarka, Nashik, India

[2]Department of Pharmacology, Shram Sadhana Bombay Trust’s Institute of Pharmacy,
Bambhori, Jalgaon, India

[3]Department of Pharmaceutics, Shram Sadhana Bombay Trust’s Institute of Pharmacy,
Bambhori, Jalgaon, India

Email: rxutkarshmandage@gmail.com

Abstract

The human gut microbiota mediates immunological, metabolic, and barrier functions, all of which have a significant impact on host health. Due to individual variability, diverse microbial diversity, and methodological variations, it is still difficult to define what makes up a "healthy" gut microbiome. This review highlights the drawbacks and difficulties of current techniques for assessing the health condition of the gut microbiome and characterizing it. In order to enhance microbiome-based diagnoses and treatments, it concludes by examining potential future developments for standardized, integrative, and functionally applicable frameworks.

Keywords

Gut microbiome, Microbiome health, Microbial diversity, Metagenomics, Multi-omics integration, Microbiome diagnostics

1. Introduction

Billions of bacteria, viruses, fungi, and archaea that make up the human gut microbiome create a dynamic and intricate ecology that is vital to host health. This microbial population influences various physiological functions, including digestion, nutrient absorption, metabolism, immune system development, and pathogen protection. The significant influence of gut microbes on health and illness has been revealed by recent developments in sequencing and multi-omics technology, making the microbiome a crucial factor in determining general well-being(1).

Consequently, finding trustworthy indicators that define a "healthy" gut microbiota is of increasing scientific and therapeutic importance. The establishment of such markers is essential for developing microbiome-based diagnostic and prognostic tools, directing personalized nutrition and therapeutic interventions, and understanding the disease etiologies linked to dysbiosis. However, because the gut microbial makeup is extremely personalized and impacted by factors including genetics, age, nutrition, lifestyle, geography, and medication usage, the idea of a single, universally healthy microbiome is still elusive (1,2).

It is extremely difficult to come to an agreement on what makes a healthy gut microbiome because of this substantial inter-individual variability. Wide variations in microbial taxa and functionality among healthy persons are shown by population-based research; these variations are made more complex by temporal oscillations within individuals over time. The definition of health-associated microbiome characteristics is also inconsistent due to methodological differences in sample collection, sequencing, and data interpretation (3).

This paper critically analyzes the existing scientific approaches used to describe and evaluate a healthy gut microbiome in light of this complexity. It discusses the restrictions and difficulties faced in this area and looks at new opportunities to enhance functional evaluation, standardization, and customized frameworks. The goal is to give a thorough perspective in order to guide future studies and clinical translation initiatives aimed at using the gut microbiota to promote health.

2. Approaches to Define a "Healthy" Gut Microbiome

There are several viewpoints that try to convey the intricacy of gut microbial ecosystems through taxonomic composition, functional potential, and clinical relevance when defining a "healthy" gut microbiome (Fig. 1).

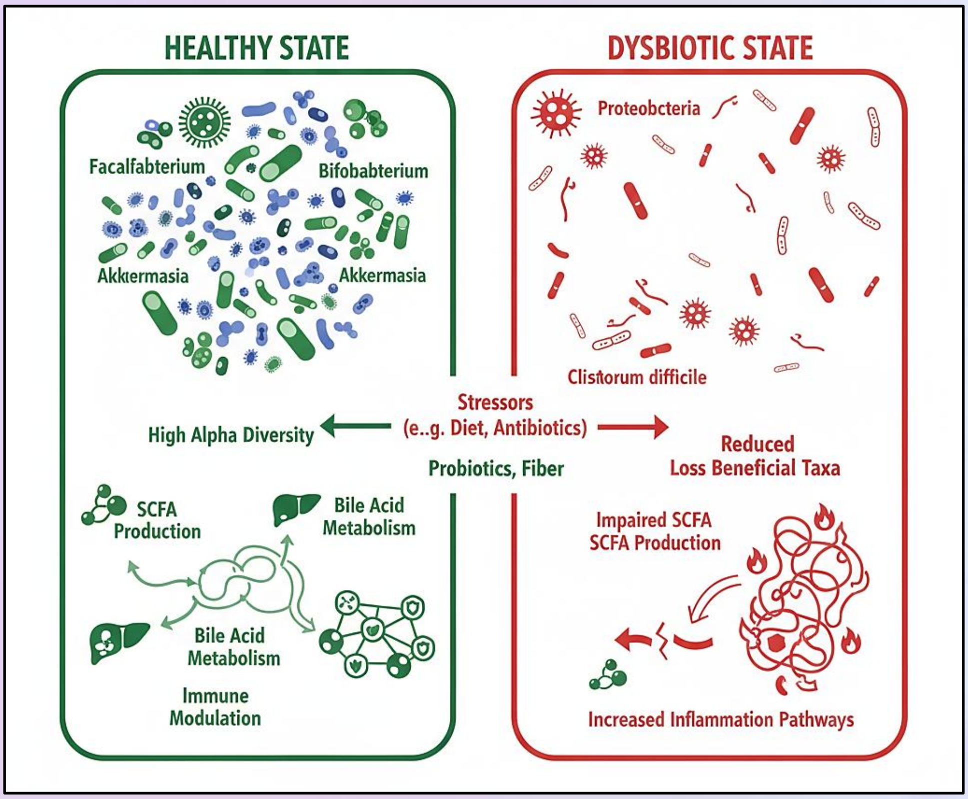


Fig 1: Gut microbiome diversity, dominant taxa, and metabolic functions in healthy versus dysbiotic states

2.1. Taxonomic and Ecological perspectives

Ecological measurements developed from community ecology are the focus of one conventional method. The two most widely used diversity indices are beta diversity, which quantifies variations in microbial composition between individuals or groups, and alpha diversity, which assesses species richness and evenness within an individual's gut microbiome.

Higher alpha diversity is typically seen to be a sign of gut health and is linked to the microbial community's stability and resilience. This association is not always true, though, as some healthy people have less diversity due to things like geography and food. The relative abundance of important bacterial phyla, including Firmicutes and Bacteroidetes, is regularly investigated at the compositional level. The Firmicutes to Bacteroidetes ratio (F/B ratio), once proposed as an indicator of metabolic health, has demonstrated variability and limited specificity. Moreover, keystone taxa, microbial species that exert a disproportionate influence on the structure and function of communities, are considered significant due to their abundance and existence. Taxa such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* have been associated with metabolic regulation and anti-inflammatory properties, rendering them potential biomarkers of gut health(1,4).

2.2. Metabolic and Functional perspectives

Researchers are increasingly emphasizing functional and metabolic parameters, recognizing that taxonomic profiles may insufficiently elucidate the connections between the microbiome and health. Metagenomic sequencing is utilized to analyze the comprehensive gene content of the microbial population to predict its metabolic capabilities. This method facilitates the identification of genes associated with beneficial processes, such as the production of short-chain fatty acids (SCFAs), which are crucial for maintaining the integrity of the gut epithelium, modulating the immune system, and providing energy to colonocytes. In addition to short-chain fatty acids, signaling pathways associated with immunological and metabolic regulation are influenced by alterations in bile acids driven by the microbiota. Functional evaluations encompass metaproteomic, which delineates expressed proteins; metabolomics, which quantifies small-molecule chemicals synthesized or modified by microbes; and meta transcriptomics, which documents gene expression levels. These multi-omics layers provide a more dynamic and comprehensive understanding of microbial activity and its relevance to host health(5,6).

2.3. Population-Based and Clinical perspectives

Multiple extensive cohort studies have linked specific microbiome compositions and functions to immunological, neurobehavioral, and metabolic health outcomes. Despite variations in outcomes due to population and methodological differences, these studies facilitate the identification of microbial signatures associated with health or disease conditions. Cross-cultural comparisons of microbiomes from diverse geographic regions and lifestyles provide valuable insights. The complexity of establishing a universally healthy microbiome is illustrated by these studies, which reveal substantial variability in microbiome composition among healthy individuals globally, impacted by nutrition, environment, and genetics. Integrative reference microbiome databases have been established to facilitate comparisons and standardize results. A prominent instance is the Human Microbiome Project (HMP), which offers comprehensive genomic and information from healthy individuals as a foundational resource. Supplementary initiatives to improve reference data comprise the American Gut Project and the MetaHIT project. These tools are crucial for analyzing and understanding the diversity of the microbiome in relation to health(5,7).

3. Methods for Measuring the Gut Microbiome

3.1. Methods Based on Culture

Culture-based techniques have historically formed the foundation for gut microbiome research by isolating and culturing bacteria on selective or non-selective media in both aerobic and anaerobic conditions. These methods enable phenotypic characterizations, functional investigations, and antibiotic susceptibility testing through the direct examination of live bacteria. Culturability is, however, restricted by the extremely complex and primarily anaerobic gut environment. Estimates show that approximately 20–30% of gut microbiota can be cultivated using traditional laboratory procedures. It can be difficult to duplicate many microorganisms in vitro because they need particular growth nutrients, symbiotic partners, or highly anaerobic environments.

As a result, culture-based techniques present a skewed picture, identifying rare, slow-growing, or uncultivable taxa while favoring the detection of abundant and rapidly developing species. The ability to fully characterize and capture the variety of the microbiome is limited by this bias. By employing high-throughput culture conditions and optimal media, recent developments in culturomics have increased the number of culturable species; nonetheless, culturomics is still time-consuming and unsuitable for routine diagnostics. Notwithstanding their drawbacks, culture techniques are useful for functional testing, isolating new species, and confirming sequencing results. These methods are still crucial for studying antibiotic resistance, developing probiotics, and comprehending the physiology of microorganisms. However, because molecular and sequencing-based techniques can identify organisms that cannot be cultured and give high-resolution community data, they have largely replaced culture for thorough gut microbiome analysis(8,9).

3.2. Methods Based on Molecular and Sequencing

3.2.1.16S rRNA Gene Sequencing

The most used molecular method for characterizing the bacterial communities in the gut is 16S rRNA gene sequencing. In order to identify bacteria taxonomically, it targets conserved portions of the 16S ribosomal RNA gene that are separated by variable regions. Compared to whole-genome techniques, this method is less computationally demanding, more affordable, and comparatively simple. It allows for high-throughput community characterization at the genus and frequently species levels.

16S sequencing does have some significant drawbacks, though. Because conserved areas across closely related species do not differ sufficiently, taxonomic resolution is frequently insufficient for species- or strain-level identification. It is limited to characterizing bacteria because it is ineffective at detecting viruses, fungi, or archaea. Additionally, primer selection, PCR amplification mistakes, and variations in reference database completeness can all lead to biases in sequencing. Furthermore, 16S sequencing does not directly yield functional information regarding the metabolism or activity of microorganisms. As a result, although useful for preliminary community surveys, alternative methods are needed to completely comprehend the functional consequences of microbiome makeup(9,10).

3.2.2.Whole Genome Shotgun Metagenomics

Whole genome shotgun (WGS) metagenomic sequencing analyzes all microbial DNA in a sample, allowing identification of bacteria, archaea, viruses, and microbial eukaryotes, typically down to strain level resolution. WGS, as opposed to 16S rRNA sequencing, reveals all of the microbial gene content, allowing for the prediction of functional traits like metabolic pathways and genes for antibiotic resistance. A systems-level understanding of the gut microbial population is made possible by the richer taxonomic and functional insights that WGS's comprehensiveness offers. It offers a more thorough representation of the microbiome ecosystem by detecting novel or previously unrecognized species and genes. WGS is costly and requires significant computational resources. Challenges include distinguishing closely related strains, unequal microbial genome coverage resulting from changing abundance, and contamination from host DNA. Precise reference databases and sophisticated bioinformatics procedures are essential for interpretation. Notwithstanding these challenges, WGS's extensive taxonomic range and functional prediction capabilities have established it as the industry benchmark for thorough microbiome characterization(2,10).

3.2.3.Metaproteomics and Metatranscriptomics

Metatranscriptomics quantifies the total RNA of the microbial population to monitor gene expression and ascertain which genes are actively transcribed under specific conditions. Our strategy provides dynamic insights into microbial functional activity rather than concentrating on static genetic potential. It elucidates the mechanisms that result in health or illness by demonstrating responses to environmental alterations, such dietary modifications or pharmacological interventions. Metaproteomics offers definitive evidence of the existence of beneficial proteins and enzymatic activities inside the microbiome by analyzing the protein expressions of gut microbes. This supports transcriptomic data by providing information on microbial metabolism and host interactions.

Both approaches require high-quality sample preservation to retain RNA and protein integrity and face complexity due to the unstable nature of RNA/protein and contamination by host molecules. Advanced mass spectrometry and sequencing technologies are necessary for analytical pipelines. Understanding real-time microbial roles and host interactions in gut health requires the use of metatranscriptomics and metaproteomics, which confirm active processes while metagenomics forecasts possible activities (11,12).

3.2.4.Metabolomics For Functional Readouts

The most direct assessment of microbiome function and its metabolic influence on the host is provided by metabolomics, which quantifies small-molecule metabolites generated or altered by gut microorganisms. Methods include mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, which are used on urine, blood, or feces samples.

Microbial contributions to the integrity of the gut barrier, the regulation of inflammation, and the metabolism of host energy are reflected in metabolites including short-chain fatty acids (SCFAs), bile acids, and amino acid derivatives. Understanding how metabolic pathways change in health and disease is made possible by profiling these metabolites.

The benefit of metabolomics is its ability to capture metabolic interactions between microbes and hosts as well as the impact of medications and food. Still, due to the complex chemical variety and overlap, the identification of metabolites can be challenging. Furthermore, outcomes are significantly influenced by sample management and external factors, complicating replication efforts. Notwithstanding its challenges, metabolomics serves as a crucial functional assessment between host physiology and microbiome structure(6,10).

3.3. Multi-omics integration

Integration of Multi-Omics Systems biology methodologies that integrate many omics layers, such as proteomics, metabolomics, transcriptomics, and genomics, offer an extensive insight into the composition and functioning of the gut microbiome. Multi-omics integration promotes resolution by correlating taxonomic identities with functional gene expression, protein synthesis, and metabolite production. This comprehensive method simulates complex microbial interactions and their effects on host pathways, providing insights into microbial ecology and the mechanisms underlying health or disease states. The integration of metagenomics and metabolomics can elucidate the relationship between gene content and active metabolic processes. Nonetheless, substantial, high-quality datasets with uniform metadata and intricate computational frameworks are essential for this type of integration. Data dimensionality, batch effects, and bringing various data kinds into harmony are among the difficulties. Despite its complexity, multi-omics systems biology is becoming more widely acknowledged as being crucial to moving microbiome research closer to therapeutic translation and mechanistic understanding(13,14).

3.4. Statistical and Computational Frameworks

Computational frameworks investigate huge microbiome datasets by utilizing statistical methods and bioinformatics. Machine learning (ML) algorithms have developed to categorize microbiome-related health conditions by identifying intricate, non-linear patterns that are beyond human detection, going beyond conventional diversity and compositional assessments. By combining taxonomic, functional, and clinical metadata, machine learning classifiers can create predictive models for therapy response or illness risk, enabling customized medical strategies. Neural networks, support vector machines, and random forests are some of the methods.

However, dataset diversity, size, and quality all affect machine learning performance. Biases and decreased generalizability result from reference databases' frequent underrepresentation of varied communities. Clinical value is limited by overfitting to training datasets and some models' inability to be biologically interpreted. To increase computational model accuracy and resilience, ongoing efforts are needed to standardize, diversify, and grow microbiome reference datasets(8,15). Table 1 summarizes the major methods for profiling the gut microbiome.

Table 1: Major Methods for Profiling the Gut Microbiome

Method	Principle/ Technology	Strengths	Limitations	Typical Applications
Culture-based	Growth on selective media	Direct study of live microbes; physiological characterization	Most gut microbes uncultivable; labor-intensive; biased toward fast-growing taxa	Isolation of novel microbes, probiotic screening, antimicrobial testing
16S rRNA Gene Sequencing	Amplicon sequencing of 16S rRNA gene	Cost-effective; high-throughput; taxonomic assignment (genus-level)	Limited taxonomic resolution; does not provide functional data; biases in primer selection	Microbial community surveys, diversity analysis
Shotgun Metagenomics	Sequencing all DNA in sample	Strain-level resolution; functional gene prediction; detects bacteria, archaea, viruses	Costly; computationally intensive; host DNA contamination; database requirements	Comprehensive taxonomic and functional profiling, detection of rare taxa
Metatranscri-ptomics	Sequencing total RNA	Reveals active gene expression; functional insights	RNA instability; complex analysis; requires deep sequencing	Assessment of dynamic microbial activity, response studies

Metaproteomics	Mass spectrometry of proteins	Direct measurement of microbial enzymes/protein function	Host protein contamination; complex sample preparation; expensive instruments	Functional profiling, host-microbe interaction studies
Metabolomics	NMR/MS-based detection of metabolites	Direct assessment of metabolic output/function	Chemical diversity; influenced by host/diet; interpretation complexity	Functional biomarker discovery, links to host physiology
Multi-omics Integration	Combined genomics, transcriptomics, proteomics, metabolomics	Holistic systems biology perspective; correlates function and taxonomy	Data integration challenges; technical complexity; large sample size needed	In-depth mechanism studies, personalized microbiome analysis

4. Limitations and Challenges

Despite tremendous progress in the study of the gut microbiome, there are still a number of inherent restrictions and difficulties in identifying and quantifying a "healthy" gut microbiome, which make both research and therapeutic applications more difficult(3,5).

4.1. Inter-individual variability

The significant inter-individual variation in gut microbiota makeup and function is one of the main obstacles. This diversity is influenced by a number of factors, including geography, ethnicity, genetics, age, and food. Infants and adults, for instance, have various microbial communities, and older people frequently have different microbial profiles and less variety. Microbial ecosystems are significantly shaped by dietary practices; various communities are favored by plant-based, fiber-rich diets as opposed to high-fat, Western-style diets. Immune regulation and host-microbe interactions are influenced by genetic background. Ethnicity and geographic location include lifestyle, cultural food habits, and environmental exposures, all of which have an effect on the microbiome. Individual or population-specific baselines are required due to this biological variety, which calls into question the creation of universal "healthy" norms(10,16).

4.2. Temporal Variability

The gut microbiota is dynamic and can change over both short and extended periods of time. While longer-term alterations are caused by dietary changes, antibiotic use, infections, or illness, daily oscillations are caused by the timing and composition of meals. It frequently takes months or years for microbial populations to recover from the severe and occasionally protracted disturbances caused by antibiotic treatments. Similar to this, gastrointestinal disorders or infections can temporarily or permanently alter microbiome patterns. The interpretation of single time-point microbiome findings is made more difficult by this temporal instability, which also emphasizes the necessity of longitudinal sampling in order to identify significant trends associated to health(1,12).

4.3. Lack of Universal Definition

A significant obstacle is the absence of an understanding over the definition of a "healthy" gut microbiome. Due to genetic and environmental factors, the traits that differentiate healthy microbiomes from dysbiotic states might differ significantly among cultures. Diagnostic and treatment criteria are complicated by the fact that what is deemed dysbiosis in one group or disease state may seem normal in another. This uncertainty prevents regulatory approval of microbiome-based therapies and restricts the clinical application of microbiome assessments(2,3).

4.4. Biases in Methodology

Biases that impact microbiome profiling are introduced by technical and methodological errors. Variability results from variations in DNA extraction procedures, storage conditions, sequencing platform selection, bioinformatic pipelines, and sample collecting techniques (e.g., feces vs. mucosal biopsy). Each stage could affect the discovery of uncommon species, skew estimates of abundance, or selectively favor or lose specific taxa. The repeatability and trustworthiness of results are challenged by platform-specific and pipeline-dependent analysis discrepancies, which make cross-study comparisons and meta-analyses more difficult(8,9).

4.5. Comparing Causation and Correlation

Without proving causation, the majority of microbiome investigations find correlations between microbial characteristics and health outcomes. It is still challenging to determine whether changes in the microbiome cause disease or are a result of secondary effects. To confirm causality, mechanistic investigations using in vitro models, germ-free animals, and clinical interventions are necessary due to the complexity of the ecosystem, host interactions, and environmental factors. Clinical translation of microbiome data is still challenging in the absence of causal proof(2,12).

4.6. Concerns about Ethics and Translation

There are ethical and translational issues when using "healthy microbiome" criteria in clinical settings. Data from a person's personalized microbiome may affect psychological effects, discrimination, and privacy. In order to avoid overinterpreting microbiome data, which are still probabilistic rather than deterministic, the notion of health must be contextually sensitive. To avoid abuse or inaccurate information, decision-making based on microbiome profiles needs strong validation. The necessity for standardized, evidence-based methods is highlighted by the ongoing evolution of clinical guidelines and regulatory frameworks for microbiome-based diagnostics and treatments(4,5).

5. Prospects for the Future

Numerous potential directions in the quickly developing field of gut microbiota and human health research give optimism for overcoming present obstacles and moving closer to practical applicability(3,6).

5.1. Moving Towards Frameworks of Universal Reference

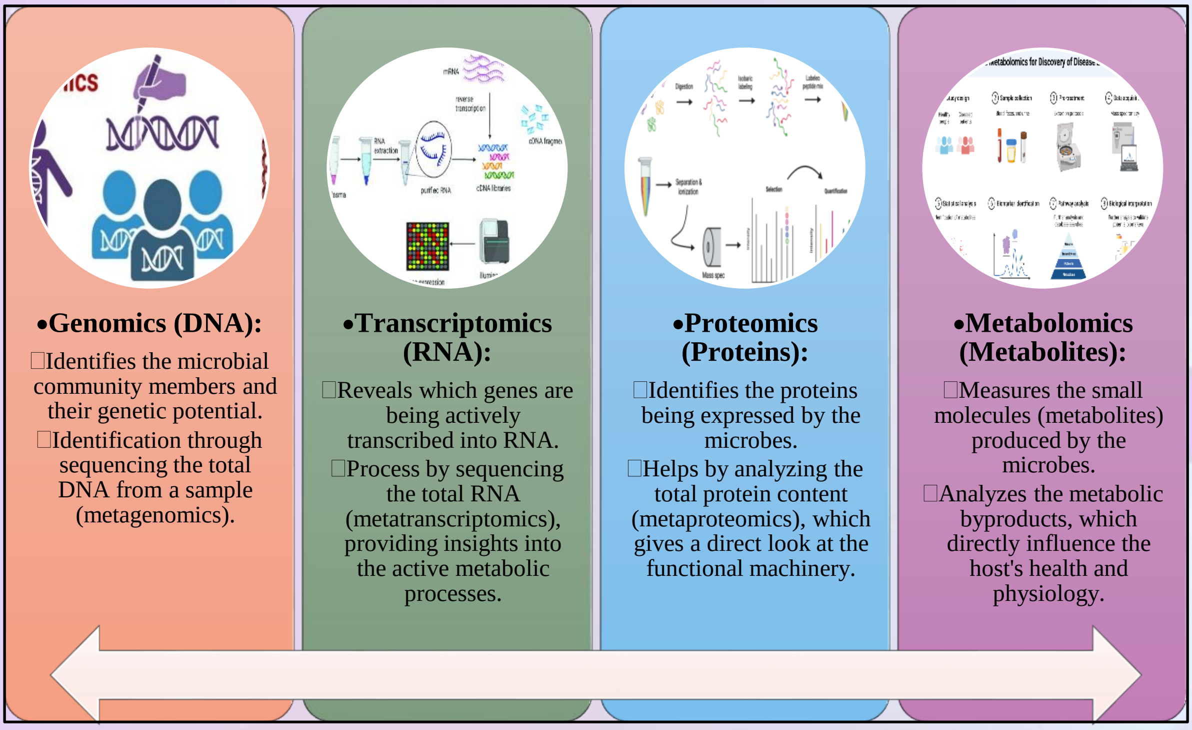
The standardization of procedures covering sample collection, storage, DNA/RNA extraction, sequencing techniques, and data analysis pipelines will be a crucial advancement. In order to reduce technological variability and enable consistent and repeatable microbiome profiles across experiments, standard operating procedures should be established. Comprehensive, well-annotated, and openly available reference databases that include a variety of populations and provide rich metadata on environmental exposures, diet, lifestyle, ethnicity, and health status are also required. The international research community will use these universal reference frames as standards for identifying "healthy" microbiome conditions and for comparing studies(16).

5.2. Personalized definitions of Microbiome Well-being

Future frameworks will place more emphasis on individualized microbiome baselines than on general population averages due to inherent inter-individual heterogeneity. These personalized references, which are a part of the rapidly developing field of precision medicine, take lifestyle, environment, food, and genetic history into consideration. By customizing recommendations to each person's own microbial signatures and trajectories, personalized microbiome health definitions will increase the specificity and sensitivity of microbiome-based diagnoses and therapies(5).

5.3. Better Platforms for Integration

Comprehensive analytical platforms that combine clinical, nutritional, and environmental metadata with multi-omics data, genomic, transcriptomic, proteomic, and metabolomic are necessary due to the intricacy of microbiome-host interactions (Fig. 2). When combined with developments in machine learning and artificial intelligence (AI), these integrative pipelines can spot minute trends that are indicative of health effects, allowing for early dysbiosis identification and dynamic monitoring. By dynamically merging taxonomic and functional data, AI models will help clinical decision-making and provide actionable health forecasts(13).



•Genomics (DNA):

Identifies the microbial community members and their genetic potential.

Identification through sequencing the total DNA from a sample (metagenomics).

•Transcriptomics (RNA):

Reveals which genes are being actively transcribed into RNA.

Process by sequencing the total RNA (metatranscriptomics), providing insights into the active metabolic processes.

•Proteomics (Proteins):

Identifies the proteins being expressed by the microbes.

Helps by analyzing the total protein content (metaproteomics), which gives a direct look at the functional machinery.

•Metabolomics (Metabolites):

Measures the small molecules (metabolites) produced by the microbes.

Analyzes the metabolic byproducts, which directly influence the host's health and physiology.

Fig. 2: Integration of genomics, transcriptomics, proteomics, and metabolomics layers for microbiome analysis

5.4. Evaluation of the Functional Microbiome

It is anticipated that future studies will place more emphasis on functional microbiome assessments and metabolic outputs and microbial activities that have direct health implications rather than just taxonomic composition. Compared to taxonomic presence alone, functional indicators including bile acid conversions, microbial enzymatic activity, and SCFA profiles may provide more insightful information about the contributions of microbes to host physiology. This functional approach provides strong targets for intervention and improves mechanistic understanding(10).

5.5. Translation in Clinical Practice

Creating validated diagnostic biomarkers that accurately reflect gut microbial health and forecast disease risk or treatment response is a crucial step in putting microbiome science into clinical practice. These biomarkers need to be clinically actionable and repeatable. Additionally, targeted dietary changes, prebiotics, probiotics, synbiotics, and modified microbial strains intended to restore or improve advantageous activities are also examples of emerging interventional microbiome therapeutics. The treatment of disorders linked to dysbiosis may benefit from fecal microbiota transplantation (FMT) and next-generation microbiota-based therapies. The incorporation of microbiome-based diagnostics and treatments into standard healthcare will be influenced by ongoing clinical trials and regulatory changes. In the end, this translational pipeline hopes to usher in a new era of microbiome-informed medicine by utilizing microbial ecosystems for individualized disease prevention, diagnosis, and therapy(3).

6. Conclusion

Due to the complex interactions between host variables, environmental influences, and microbial community dynamics, defining and quantifying a "healthy" gut microbiome continues to be a challenging and dynamic task. Current approaches that combine ecological, taxonomic, and functional viewpoints offer insightful information about the health of the gut microbiome, but they are frequently lacking and occasionally contradictory. Efforts to create universal benchmarks are complicated by the high inter-individual and temporal variability, methodological flaws, and the challenge of proving causation.

However, developments in computational modeling, multi-omics integration, and sequencing technologies are steadily improving our knowledge of the gut microbiome's function in health. In the future, the focus will be on functionally informed and tailored frameworks, which have the potential to produce more precise and clinically applicable definitions of microbiome health. To overcome existing restrictions, it will be crucial to expand a variety of reference datasets, standardize procedures, and integrate artificial intelligence.

Crucially, the successful restoration or maintenance of a healthy microbial ecosystem depends on validated indicators and focused therapies in order to translate microbiome science into clinical practice. Although there are still obstacles to overcome, new multidisciplinary strategies that combine clinical sciences, analytics, and microbiology point to a time when the gut microbiota will play a key role in preventative healthcare and personalized therapy.

In summary, it is preferable to think of the idea of a "healthy" gut microbiome as a dynamic, unique, and context-dependent condition. Important microbiome-based diagnostics and treatments will eventually improve human health thanks to ongoing research efforts focused on standardizing methodology and expanding functional insights.

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