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Clinical Microbiome Analysis by Mass Spectrometry–Based Metaproteomics

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Abstract

Mass spectrometry–based proteomics and metaproteomics have long been used in the study of human microbiomes, with the potential of metaproteomics only recently being fully harnessed. This progress is due to the advancements of high-performance mass spectrometers, innovative proteomics strategies, and the development of dedicated bioinformatics tools. In this review, we critically examine the recent technological developments that enhance the application of metaproteomics in clinical microbiome analysis. We also summarize significant advancements in the application of metaproteomics to study human microbiomes across various body sites under disease conditions. Despite these, the potential of metaproteomics remains underutilized due to typically small sample sizes and insufficient data mining. We thereby highlight some key aspects that could facilitate the broader and more effective application of mass spectrometry–based metaproteomics in clinical microbiome analysis, including the development of microbiome assays for translational research and application.

1. INTRODUCTION

The human body is colonized by trillions of microorganisms, including bacteria, viruses, fungi, archaea, and protozoa, collectively referred to as the human microbiome. These microorganisms are present on nearly all surfaces of the human body. Extensive research has been conducted on the bacterial portion of the human microbiome, but other components have also been shown to be critically important. They can reshape bacterial composition or directly interact with human cells (1). The human gut microbiota is the most well-characterized to date, but microbiomes from other body parts, such as the skin, oral cavity, and urogenital tract are increasingly being investigated and have been reported to impact human health both locally and systemically (2).

In the past two decades, accumulating evidence has revealed the association and even causative roles of human microbiomes in various diseases. The human gut microbiome has been shown to be involved in diseases ranging from gastrointestinal disorders and metabolic conditions to cardiovascular diseases, neurological disorders, and cancers (3). Similarly, the skin microbiome is often implicated in atopic dermatitis (4), while dysbiosis of the urogenital microbiome is commonly associated with urinary tract infections (UTIs) and bladder cancer, as well as preterm birth and abnormal discharge in pregnant women (5, 6). During the COVID-19 pandemic, the role of the respiratory microbiomes in respiratory infections and diseases, such as cystic fibrosis and chronic obstructive pulmonary disease, has become increasingly evident (7–9). Altogether, these findings underscore the importance of the symbiotic relationship between humans and their microbiomes in maintaining health.

Accordingly, therapeutic treatments and dietary interventions targeting the microbiome were developed for treating diseases, including *Clostridioides difficile* infection (CDI), type 2 diabetes, inflammatory bowel disease (IBD), and obesity. One of the most successful examples is fecal microbiota transplantation (FMT), which showed significant clinical benefits in preventing the recurrence of CDI following antibiotic treatments (10). To date, at least three FMT products (REBYOTA™, VOWST™, and BIOMICTRA™) are approved for treating CDI. Meanwhile, more than a thousand clinical trials are ongoing worldwide to test FMT for various diseases, including cancers, IBD, and metabolic syndromes (**Figure 1**). As microbiome research advances toward more translational and functional investigations, an increasing number of microbial or associated metabolic biomarkers will be identified, further facilitating the microbiome-directed precision medicine and wellness care (3). While targeted biomarker analysis is common in clinical practice, the analysis of whole microbiomes using modern bioanalytical methods in a quick and cost-effective manner is important for biomarker discovery or direct clinical applications, addressing the complex mechanisms of action in host–microbiome interactions.

To date, most microbiome studies have utilized nucleic acid sequencing techniques, including 16S ribosomal RNA (rRNA) gene amplicon sequencing-based metataxonomics and shotgun DNA/RNA sequencing-based metagenomics or metatranscriptomics. These methods are favored due to their high depth of measurement, which includes detection of low-abundance species, and their lower cost with high multiplexing capacity, particularly for marker gene sequencing approaches. However, as the microbiome research field evolves, functional analysis of the microbiomes has become increasingly important for studying expressed functions and metabolic outputs, rather than solely profiling taxonomic composition and functional potentials (3). Mass spectrometry (MS)-based metaproteomics and metabolomics offer the advantage of directly measuring expressed proteins, post-translational modifications (PTMs), and metabolic outputs that interact with the host (11). As a result, these technologies are increasingly applied to microbiome research, thanks to significant advancements in the field. The MS instruments have evolved rapidly, with improvements in sensitivity, scanning speed, and resolution (12, 13). These improvements have enabled the high-throughput, high-depth, and rapid analysis of microbiomes

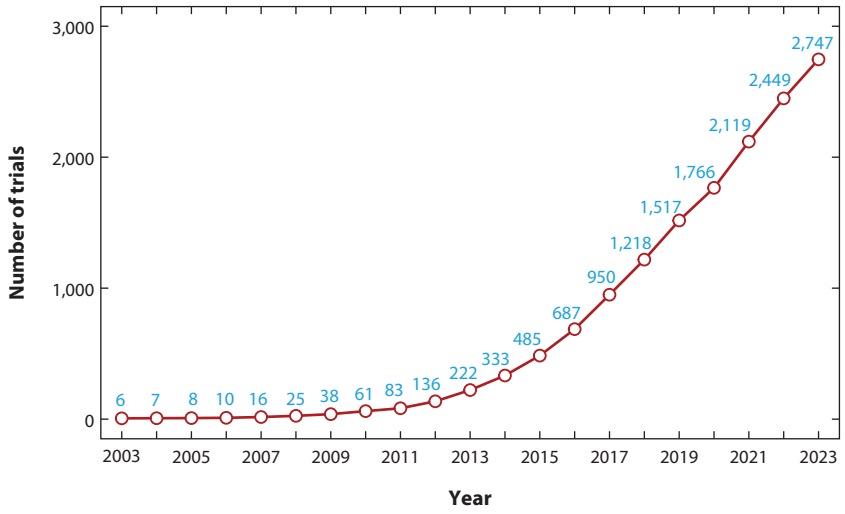

Figure 1

Number of clinical trials in the past 20 years with a focus on the microbiome. The trial records in ClinicalTrials.gov were searched on June 28, 2024, with the parameter [Title and/or Title Acronym: "microbiota" OR "microbiome" OR "microbial community"]. Only clinical trials that were started between 2003 and 2023 are included.

with ultra sensitivity. Sample preparation has been automated (14), and in-depth metaproteomic sample analysis can now be completed in as little as a few minutes (15). Additionally, dedicated bioinformatics tools or workflows are actively being developed to facilitate the rapid interpretation of acquired MS data, providing taxonomic and functional information of microbiome samples (16, 17). Overall, the analysis of clinical microbiomes with MS is emerging as a crucial aspect of health research and holds promise for wide clinical applications.

In this review, we first examine the technological developments and challenges of using MS for analyzing proteins in clinical microbiome samples. We then summarize the current applications of metaproteomics for analyzing microbiomes from various types of clinical samples in the context of human disease research. Additionally, we showcase recent applications of MS-based methods in facilitating microbiome assay development and their potential for facilitating precision disease treatment. Overall, this review aims to highlight the unique capabilities of MS-based modern bioanalytical methods in deciphering host–microbiome interactions and their potential applications in clinical settings.

2. TECHNICAL ADVANCEMENT OF METAPROTEOMICS

Metaproteomics has emerged as a powerful technique for unraveling the complexities of microbiomes. Key developments in metaproteomics that, we believe, are going to transform the field include (*a*) higher-throughput and robust sample processing workflows, (*b*) data-independent acquisition mass spectrometry (DIA-MS), and (*c*) highly efficient software tools for metaproteomics statistical and bioinformatic analysis (**Figure 2**).

2.1. Metaproteomic Sample Preparation

Due to the complexity of microbiome samples, which encompass various species and proteins, two key principles must be adhered to: reducing bias and enhancing sensitivity. These principles

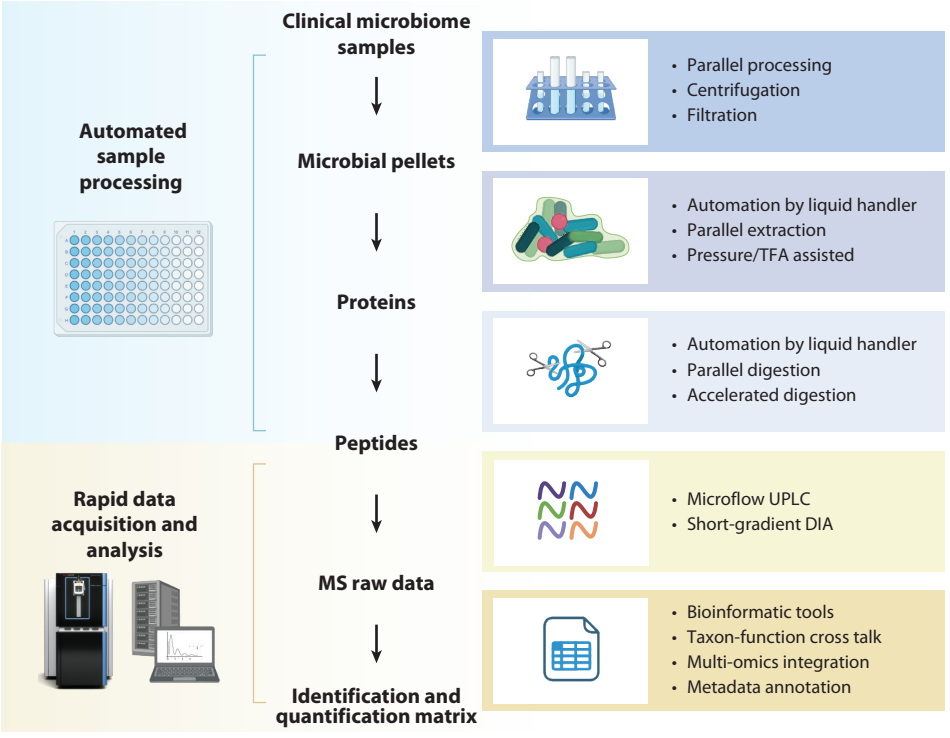

Figure 2

Key aspects of metaproteomics workflow for clinical microbiome analysis. The figure highlights key considerations and advancements of metaproteomics in recent years that may boost its application in clinical microbiome analysis. Abbreviations: DIA, data-independent acquisition; MS, mass spectrometry; TFA, trifluoroacetic acid; UPLC, ultraperformance liquid chromatography. Figure adapted from images created with BioRender.com.

ensure a more accurate representation of the microbial profile. Clinical microbiome samples, characterized by significant individual variations, contain proteins from both host cells and microbial communities, as well as confounding factors such as food debris. Researchers face challenges in preprocessing and efficiently extracting proteins from clinical microbiome samples due to low-abundance proteins, dynamic range, and sample heterogeneity. Enrichment of microbial cells from microbiome samples can improve protein identifications but risks losing important components (e.g., host-secreted proteins) (18, 19). Different protein extraction methods yield distinct protein profiles, complicating comparisons across labs and experiments at higher taxonomic and functional levels. Previous evaluations suggest that mechanical disruption, combined with a strong lysis buffer, is crucial for breaking the cell wall and achieving better protein yields for gram-positive bacteria in human gut microbiome (20).

Another challenge for metaproteomics application in clinical microbiome analysis is the need for high-throughput and rapid turnaround. The recently developed SPEED (Sample Preparation by Easy Extraction and Digestion) protocol shows great promise for metaproteomics as well (21). It offers good extraction and digestion efficiency for various bacterial cells, with the entire process (from protein extraction to clean peptides) completed in hours due to simultaneous reduction by *tris*(2-carboxyethyl)-phosphine (TCEP) and alkylation by chloroacetamide and high-temperature digestion.

Automated liquid handling systems are streamlining sample preparation workflows, reducing human error, improving reproducibility, and allowing for higher-throughput sample preparation for metaproteomics. The single-pot, solid-phase-enhanced sample preparation (SP3) method has become the gold standard for one-pot sample preparation owing to its compatibility with detergents and automation (22). An autoSP3 method was developed in a 96-well plate format for unbiased protein purification and digestion, with peptide samples ready to load to liquid chromatography (LC)-MS for analysis (23), which showed great reproducibility and robustness with clinical samples. A solvent precipitation SP3 (termed SP4) achieves protein recovery through acetonitrile-induced protein aggregation and centrifugation instead of magnetic capturing, which was recently demonstrated to be a robust proteomic sample preparation method (24). Technically, the SPEED method mentioned above is more automation-friendly, requiring fewer steps and no centrifugation or magnets or special equipment.

These advancements in sample preparation are vital for unlocking the full potential of metaproteomics. By minimizing bias, enriching microbial proteins, and ensuring efficient protein extraction and digestion, researchers are obtaining clearer insights into the functional protein profiles in microbiome samples for both the host and microorganisms. This information enhances our understanding of their roles in various ecosystems and their impact on human health.

2.2. Mass Spectrometry Data Acquisition

In high-throughput clinical proteomics studies, LC-MS analysis often represents significant constraints related to performance consistency and time usage. To address these challenges, there has been a shift toward reintegrating microflow rate high-performance liquid chromatography (HPLC) with MS from nanoLC-MS (25). This approach offers improved consistency and stability, reduced LC dead volume, and more effective use of data acquisition time. Although there is a trade-off in sensitivity, this limitation can be effectively mitigated by incorporating a 5% DMSO solution into the buffer system (26).

For MS data acquisition, DIA mode has gained increasing attention, gradually replacing data-dependent acquisition (DDA). DIA is revolutionizing metaproteomics by effectively handling the complexity and analyzing low abundant species, which overwhelms traditional DDA workflow due to the limited MS scanning speed within a defined LC gradient time (27). In DIA, all precursors are fragmented, and the fragmentation data are integrated into retention time-modulated chimeric spectra (28). This approach has led to the identification and quantification of up to 70,000 microbial proteins in a single metaproteomic study (29).

Off-line protein- or peptide-level fractionation can enhance metaproteomic depth, particularly in DDA workflows (30, 31); however, it is not cost-effective for clinical metaproteomics and risks losing quantitative accuracy. DIA increases proteome depth while maintaining quantitative precision, a feat often considered impossible with DDA workflows (32). A significant recent advancement in MS instrumentation is the Orbitrap Astral, which supports fast (up to 200 Hz) narrow window (2 Da) DIA-MS, bridging the gap between DDA and DIA (12, 33). This technology has achieved the identification of up to 122,000 unique peptides and 38,000 protein groups within a 30-min LC-MS run from a microbiome sample (15).

Multiplexing is an important way to increase the throughput of sample analysis. Currently, MS1-based multiplexing can be integrated with DIA to create plex-DIA, enhancing throughput and increasing identification confidence through the use of doublets or triplets (34). The complexity introduced by MS1 multiplexing should not pose a problem with narrow window DIA-MS workflows. However, the scale is constrained by the MS1 multiplexing capacity, which is currently limited to three [as per the proof-of-principle study (34)]. Looking ahead, we anticipate

that NeuCode labels, which can support up to 18-plex or 35-plex (35), will be combined with DIA to further boost throughput.

Tandem mass tag (TMT)-based multiplexing has been applied to both proteomics and metaproteomics to increase the throughput of sample analysis (36). Unfortunately, combining DIA with TMT techniques is not yet feasible because TMT relies on MS2-level quantification. Multiplexing for DIA must be nonisobaric because, once fragmented, the isobaric quantification channels overlap, losing the mapping between the precursor and the channels (34). This incompatibility between TMT and DIA poses a tough choice for quantification methods in large-scale clinical metaproteomic studies with hundreds to thousands of samples: TMT or DIA? Given the complexity of metaproteome samples and recent advancements in DIA, particularly narrow-window DIA on Orbitrap Astral, it has been shown that DIA outperforms TMT in applications for microbiome studies (29). This superiority of DIA can be due to the following reasons. (*a*) Complexity and coelution: Metaproteome complexity causes significant coelution of peptides with similar mass-to-charge (*m/z*) (37). In TMT's narrow isolation window, more precursor ions are selected for fragmentation, leading to severe ratio compression and chimeric issues, complicating peptide identification. (*b*) Fewer missing values: Block- or run-wise missing values are detrimental at scale. In DDA, precursors can be partially recovered by match-between-runs (38) or spectral clustering (39), while DIA typically has fewer missing values, usually less than 5% (40). Moreover, missing values in DIA are sample-wise rather than block-wise, making imputation easier if needed. (*c*) Higher throughput: Microflow rate HPLC allows for processing up to 300 samples per day via DIA on Orbitrap Astral, with more identifications compared to TMT within the same instrument time (12). Lastly, (*d*) lower cost: The cost of TMT reagents scales with the sample size, while no additional reagent costs are associated with DIA-MS, making DIA a more cost-effective option for large-scale clinical proteomics or metaproteomics studies.

2.3. Bioinformatics and Statistical Workflows

The integrated human gut microbial gene catalog (IGC) database contains 9.9 million genes (41), which leads to few protein identifications using conventional proteomic database search strategies with standard false discovery rate cut-offs (42). Recently, we and others have developed techniques and software tools to perform metaproteomic data analysis, including bioinformatic and statistical methods. We released MetaLab for metaproteomic data analysis in 2017 (43) for DDA-based experiments using a two-pass strategy to first decrease the size of the database and then search against the reduced database for identification and quantification (44). In 2020, an updated MetaLab 2.0 capable of PTM analysis on a metaproteome scale was released (45). Using MetaLab, we reported an ultradeep metaproteomic analysis of approximately 30,000 unique microbiome proteins, allowing the characterization of pathways, sometimes at the bacterial strain level (30). In 2023, we introduced MetaLab-MAG (46), which enabled the analysis of metaproteomics data at the species and strain level, leveraging the MGnify biome-specific genome databases (47). In 2022, we released MetaProClust-MS1 (48), a tool to analyze the features observed in MS1. The results indicate that a large number of microbiome-derived peptides are not identified by DDA-based approaches but are within the analytical detection range of mass spectrometers (27). This limitation led us to curate a MetaPep database consisting of all identified quality peptide sequences from human metaproteomes to date (49), which can accelerate DDA data analysis for high-throughput experiments and the development of DIA-based metaproteomics.

In contrast to DDA, DIA uses a set of precursor isolation windows to collect all fragment ions indiscriminately. It is a game changer in proteomics and has demonstrated remarkable robustness, sensitivity, and reproducibility with fewer missing values (50, 51). The development of

DIA-specific searching software tools, such as DIA-NN (52), MaxDIA (53), and Spectronaut (54), has been key to enable the analysis of DIA datasets. Readers are recommended to refer to these articles (55, 56) for a comprehensive review of the current software for DIA data.

Despite the advantages of DIA in proteomics, its full benefits have not yet been realized in the field of metaproteomics. The inherent complexity of DIA data necessitates a more constrained search space compared to DDA. Early attempts to utilize DIA for metaproteomics involved using spectral libraries derived from DDA data (16, 57, 58). However, this approach has several drawbacks: (*a*) It requires both DDA and DIA analyses of the samples, and (*b*) a significant portion of peptides present in a sample are not identified by DDA and thus are absent from the spectral libraries. These drawbacks limit the DIA to only those parts of the peptides that can be identified by DDA. To address these limitations, a method called glaDIAtor was introduced, which analyzes DIA data for microbiome samples without requiring a DDA-based spectral library (59). However, it still relies on spectrum-centric algorithms and does not fully exploit the potential advantages of DIA data. We have recently released MetaLab-DIA (**https://www.imetalab.ca**), an advanced version of MetaLab designed to process DIA-MS data without the need for constructing a library from previous DDA experiments.

Another important aspect of metaproteomics is downstream taxonomic and functional analysis. While several tools such as Unipept (60, 61), MetaLab (43, 45, 46), and PathwayPilot (62) have been developed for this purpose, linking taxon and function in metaproteomics remains challenging. In the human gut microbiome, approximately 200 microbial species are present at any given time, with considerable variation between individuals. This complexity means that for each peptide identified, the phylogenetic profile, its abundance, and the link to proteins and taxa must be maintained for post-MS analysis. Consequently, bioinformatic tools for metaproteomics must be flexible, allowing data and their relationships to be explored at different levels (i.e., peptide, taxa, taxa-function, etc.). Proteome-level functional redundancy analysis provides a new angle into the metaproteomics data (31). We recently released a beta version of MetaX, a comprehensive and flexible peptide-centric tool for analyzing taxa-function cross talk through operational taxon-function (OTF) units in metaproteomics (63).

Altogether, newly developed DIA-MS technologies, advanced software tools, and recent MS instruments are changing the way we perform metaproteomics, achieving remarkable depth and sensitivity of measurements. Several clinical metaproteomics studies leveraging DIA-MS have been published recently (16, 64, 65). Although these studies have small sample sizes, they demonstrate the significant advantages of DIA-MS approaches in clinical microbiome research.

3. APPLICATIONS OF METAPROTEOMICS IN HUMAN DISEASES

Over the past decades, a significant number of publications have reported on studies using MS-based metaproteomics to analyze various types of clinical microbiome samples. Rather than providing an encyclopedic overview of all these studies, we illustrate the technology and its scope of application by highlighting a limited selection of representative papers from the past five years.

3.1. Fecal Microbiomes

The fecal microbiome is the most extensively studied due to the fact that fecal sample collection is noninvasive and the gastrointestinal tract is the most densely colonized area in the human body by microorganisms. Accordingly, most current metaproteomics studies on human microbiomes utilize fecal samples.

Early fecal proteomics studies primarily focused on host proteins due to the technical challenges as described above. These limitations were significantly mitigated around a decade ago

when several human microbiome projects were completed (generating reference gene or genome databases for human microbiome), and new bioinformatics strategies and tools were developed. Nevertheless, the capability to identify both host proteins and proteins from all other kingdoms of microorganisms, including viruses, archaea, and fungi, is one of the key advantages of metaproteomics, compared to DNA sequencing-based metagenomics or metatranscriptomics. As a result, metaproteomics is increasingly applied in clinical microbiome analysis, and potential fecal protein biomarkers (both microbial and host origin) have been identified in various conditions, including IBD (66, 67), obesity (68), neurological disorders (69), chronic kidney disease (70), and preterm birth (71).

However, the majority of current metaproteomic studies have been limited by small sample sizes, likely due to the high cost of instrumentation. Since 2018, an increasing number of metaproteomics studies have included medium-sized sample groups (50–100 patients or approximately 200 MS samples) to enhance the confidence and biological relevance of metaproteomic observations. For instance, by analyzing more than 100 stool samples from healthy controls, new-onset type 1 diabetes patients, and high- and low-risk prediabetes patients, Gavin et al. (72) identified potential fecal protein biomarker signatures (including exocrine enzymes and microbial proteins) that can be used to monitor disease progression in type 1 diabetes. Zhong et al. (73) analyzed more than 250 fecal samples from type 2 diabetes and prediabetes patients using both metagenomics and metaproteomics, identifying an enrichment of *Escherichia coli* at both the protein and DNA levels, as well as an increase in the levels of fecal antimicrobial peptides and pancreatic enzymes in type 2 diabetes patients.

In theory, metaproteomics alone can provide both taxonomic and functional information about microbiome compositions, as genetic information is retained in the amino acid sequences of proteins. However, historically, many metaproteomics studies have been performed in combination with metagenomics. Efficient integration of multiple meta-omics data can offer detailed insights into host–microbiome interactions, identifying who is doing what with which mechanisms. Heintz-Buschart et al. (74) established a reference genome-independent workflow for integrating metagenomics and metatranscriptomics with metaproteomics in a familial type 1 diabetes cohort. This analysis demonstrated that metaproteomic profiles tend to correlate more closely with metatranscriptomic profiles than with metagenomic profiles and revealed that type 1 diabetes-specific functions (e.g., reactive oxygen species detoxification genes) were often carried out by genomes that did not show different abundances in the patients. An integrated multi-omics approach, including metaproteomics, was also applied in the integrative human microbiome project (HMP2 or iHMP). In the IBD project of iHMP, 450 metaproteomic samples were analyzed, representing one of the largest metaproteomics studies of clinical microbiome samples to date (75). However, the analysis of the metaproteomics data was limited in this study. There remains a need for large-scale metaproteomics studies to showcase its benefits and unique capabilities for clinical microbiome analysis.

Metaproteomics offers valuable functional insights beyond protein expression levels. These include the identification and quantification of low-abundance PTM proteins through PTM-specific enrichment and the profiling of enzyme activities using activity-based probe profiling (ABPP). Our previous studies demonstrated the identification of more than 60,000 lysine acetylated and approximately 20,000 propionylated and succinylated microbial peptides from human fecal microbiomes, achieved through lysine acylation motif antibody enrichment (76). Applying this approach to study microbiome samples from pediatric Crohn's disease patients revealed alterations in lysine acetylation levels in both host immune and microbial proteins, particularly those involved in translation and carbohydrate metabolism originated from known short-chain fatty acid (SCFA) producers (77). In a fecal metaproteomic analysis comparing ulcerative colitis (UC)

patients to healthy individuals, Thuy-Boun et al. (78) uncovered a significant increase in microbial serine-type endopeptidase abundance associated with UC. Employing a serine-reactive probe ABPP, they validated the presence of active serine-type endopeptidase activity of both host and microbial origin. Additionally, they identified previously undetected endopeptidases that could play a role in UC pathogenesis.

3.2. Intestinal Aspirate Microbiomes

One advantage of fecal sampling is its noninvasive nature. However, the human fecal microbiome represents a mixture of microbiomes from the entire gastrointestinal tract and cannot provide in situ conditions at the site of disease. Different segments of the gastrointestinal tract have distinct microbial compositions and functionalities. To address this challenge in human microbiome studies, Li et al. (79) introduced the mucosal-luminal interface (MLI) aspirate or lavage sampling method during diagnostic colonoscopic examinations. Using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, they analyzed 205 MLI lavage samples from 38 healthy individuals and identified biogeographic-specific host and microbial protein distributions (79). With a 16S rRNA gene sequencing approach, Mottawea et al. (80) also demonstrated that MLI sampling is ideal for obtaining region-specific, biopsy-like microbial compositions in pediatric patients. Similarly, Folz and colleagues (81, 82) conducted a metabolomics study on 15 healthy individuals with nearly 300 intestinal samples collected with an ingestible sampling device, revealing dramatic differences between stool and intestinal metabolomes, with the microbially linked MLI metabolites accounting for the largest interindividual differences. These studies collectively demonstrate that MLI sampling is a promising method for assessing human gut microbiomes.

IBD is one of the most studied diseases using MLI sampling so far, as colonoscopy is routinely used for diagnostic purposes. In an early study by Presley et al. (83), surface-enhanced laser desorption/ionization TOF (SELDI-TOF) or MALDI-TOF MS was used to identify MLI proteins and bacterial phylotypes [with sequence-selective quantitative polymerase chain reaction (qPCR)], distinguishing IBD patients from healthy controls, particularly at the sigmoid colon. More recently, they extended their study to larger patient cohorts. By sampling at six locations in 51 IBD or control patients, Li et al. (84) performed metaproteomics analysis on 257 MLI samples with MALDI-TOF MS, identifying nine robust microgeographic-specific and IBD-associated MLI protein modules using network analysis. These microgeographic protein reorganizations in the gut of IBD patients provide unique insights into disease pathogenesis at specific sites.

We have applied high-resolution Orbitrap MS-based proteomics and metaproteomics to study MLI microbiome and host proteins in pediatric IBD patients. Deeke et al. (85) used a quantitative proteomics approach to study host proteins in MLI samples from pediatric IBD patients. They identified a panel of four MLI human proteins (leukotriene A-4 hydrolase, catalase, transketolase, and annexin A3) as potential biomarkers for active IBD and a set of four MLI human proteins (leukotriene A-4 hydrolase, thioredoxin domain-containing protein 17, thymosin β-10, and vasodilator-stimulated phosphoprotein) that successfully differentiated pancolitis from non-pancolitis UC. Zhang et al. (86) performed a comprehensive metaproteomic characterization of MLI bacterial, archaeal, fungal, viral, and host proteins in 176 samples collected from 71 pediatric IBD patients and controls. Their study revealed that host-derived extracellular vesicles with antimicrobial cargo proteins play an important role in mediating dysbiotic host–microbiome interactions in pediatric IBD. Li et al. (87) combined MALDI-TOF MS and porous graphitized carbon nano-LC-tandem MS (MS/MS) methods to characterize N-glycans in MLI samples

from 73 pediatric new-onset UC patients and controls. Their findings demonstrated that pediatric UC patients had elevated levels of microbiota-associated paucimannosidic and truncated N-glycans.

In addition to IBD, MLI sampling has been applied to other diseases, such as colorectal cancer. As an example, Tanca et al. (88) performed metaproteomics on MLI samples from a cohort of 24 colorectal cancer patients at different stages and disease grades, identifying microbial taxa such as *Bifidobacterium* and *Bacteroides fragilis*, as well as metabolic functions like formate–tetrahydrofolate ligase, which were elevated in high-stage colon cancer.

Given the increasingly recognized benefits of sampling intestinal content for microbiome studies, efforts have been made to develop ingestible devices that can reach different intestinal segments for examination and sampling. Accordingly, using such a disposable ingestible capsule device, researchers collected approximately 300 intestinal samples from 15 healthy individuals and performed multi-omics profiling, demonstrating distinct spatial microbiome compositions, metabolite profiles, and host protein expressions along the gastrointestinal tract (81, 82). These sampling strategies represent significant technological breakthroughs in recent years for microbiome studies. More details on this topic are reviewed in a recent paper by Rehan et al. (89).

3.3. Respiratory Microbiomes

The respiratory microbiome is important for human health, particularly due to the recent COVID-19 pandemic. Various sample types can be collected to study respiratory microbiomes, including nasopharyngeal and oropharyngeal swabs, sputum, and the more invasive bronchoalveolar lavage (BAL) (90). While microbiome sequencing remains the primary technique for studying respiratory microbiomes, MS-based metaproteomics have been applied in some respiratory diseases such as cystic fibrosis and COVID-19.

Cystic fibrosis is a multisystem disorder characterized by the accumulation of thick mucus layers in different organs, including the lungs. BAL samples have traditionally been used to study respiratory microbiomes in cystic fibrosis patients. For metaproteomics, Kruk et al. (91) very recently established a workflow combining untargeted shotgun metaproteomics and targeted parallel-reaction monitoring (PRM) to study BAL microbiome samples. They identified host and microbial features that are known to associate with cystic fibrosis (e.g., host neutrophil elastase and collagenase and microbial pathogen *Pseudomonas*), as well as potentially novel host–microbiome features (91).

A disadvantage of BAL is that it is an invasive sampling approach, which limits its wide application in large-scale respiratory microbiome studies. Sputum is another sample type used to study cystic fibrosis microbiomes. Although no large-scale sputum sample analyses have been conducted using MS-based metaproteomics due to the high inhomogeneity, high viscosity, and low levels of microbial cells, efforts have been made to establish and optimize sample preparation methods for sputum metaproteomics (92, 93). Preliminary analyses have demonstrated the benefits of using metaproteomics to provide functional information on sputum microbiomes, offering a complementary view to culture-based studies that are commonly used in sputum microbiological studies or testing.

COVID-19 has claimed millions of lives worldwide over the past four years. Recently, researchers started to employ multi-omics approaches to study respiratory microbiomes to better understand how SARS-CoV-2 infection impacts human health. Bihani et al. (94) established an experimental and bioinformatic workflow for metaproteomic analysis of nasopharyngeal swab samples from COVID-19 patients. They found that microbial proteins from several opportunistic pathogens related to stress response and DNA damage were upregulated in severe

COVID-19 patients (94). Maras et al. (8) conducted a multi-omics analysis of combined oropharyngeal and nasopharyngeal swabs from COVID-19 patients, including proteomics, metaproteomics and metabolomics. They identified and validated potential lung protein biomarkers for COVID-19, such as MX1 (MX dynamin-like GTPase 1) and WARS (tryptophan-tRNA ligase). They also demonstrated the elevation of microbial proteins from potential coinfected pathogens and altered metabolomes, such as *N*-acetylserotonin and azelaic acid, in COVID-19 patients compared to healthy individuals (8).

3.4. Urogenital Microbiomes

Urogenital diseases are among the most common diseases worldwide, affecting the human urinary and genital systems. Following the success of gut microbiome studies, more researchers have begun to investigate urogenital microbiomes in the context of urogenital diseases such as UTIs, kidney disease, bladder infections, prostatitis, and vaginitis (5, 95). Urogenital microbiomes can be studied using urine and genital swab samples. Like other human microbiomes, DNA sequencing is commonly used, but MS-based metaproteomics also have been applied to understand urogenital microbial and metabolic alterations in recent years.

Urine sediment has been commonly used for proteomic analysis due to its high protein and microbial contents (96). Yu et al. (97) performed a proteomic analysis on 120 urine sediment samples collected from UTI patients to evaluate the feasibility of using proteomics for diagnostic purposes. They demonstrated that proteomics allows quantification of urine host proteins that correlate well with neutrophil response and urothelial injury; they also identified microbial proteins with equivalent diagnostic accuracy compared to the standard urine culture method. Re-analysis of the dataset used a new bioinformatics workflow (i.e., ProteoStorm) with a more comprehensive protein sequence database of identified known pathogens (e.g., *E. coli*, *Klebsiella*, *Pseudomonas*) and polymicrobial patterns and previously unidentified infections by *Propionimicrobium* in UTI patients (98).

Vaginal microbiome study with metaproteomics can be performed with samples, such as vaginal swabs, Pap test fluid, and cervicovaginal lavage (99–101). By using a two-step database search strategy, Afiuni-Zadeh et al. (100) demonstrated the feasibility of identifying 300 bacterial or fungal peptides from residual cell-free fixatives from discarded Pap test samples. Database selection is a major hurdle for metaproteomics of underinvestigated microbiomes, such as the vaginal microbiome. Lee et al. (99) evaluated six different database construction strategies and demonstrated that a hybrid approach (containing reference genomes and translated bacterial genes from metagenome) performs the best for metaproteomics of cervicovaginal lavage samples collected from vaginosis or control patients. The optimized workflow identified previously known proteins altered in vaginosis. With metaproteomics, Alisoltani et al. (101) demonstrated that vaginal microbial proteins perform better than taxonomic composition based on 16S rRNA gene sequencing in predicting vaginal inflammatory cytokine profiles in 113 young South African women at high risk of HIV infection. In particular, the *Lactobacillus* protein expression levels of cell wall organization and peptidoglycan biosynthesis pathways were decreased in women with high vaginal inflammation.

3.5. Oral and Other Microbiomes

Oral microbiomes are linked to various oral diseases, including caries and periodontitis. Similar to gut microbiomes, oral microbiomes exhibit high interindividual variation (102). Changes in the diversity of oral microbiomes can initiate and establish oral infections (103). Saliva or mouth rinse samples are commonly used noninvasive methods to study oral microbiomes. Additionally,

samples can be collected via buccal swabs or gingival samples, the latter being particularly useful for studying dental diseases such as caries and periodontitis (7, 104).

There are limited metaproteomics studies of oral microbiomes so far, likely due to the lack of well-established workflows, particularly for bioinformatic analysis. However, recent efforts have made progress in this area. For instance, Thuy-Boun et al. (9) developed a metaproteomic data analysis workflow using a previous proteomic dataset of gargling solution samples, detecting opportunistic pathogens in COVID-19 patients. Jiang et al. (105) used free-flow isoelectric focusing electrophoresis-mass spectrometry (FFIEF-MS) to perform a deep metaproteomic characterization of oral microbiomes in lung cancer patients, revealing dysbiosis, including elevated *Fusobacterium nucleatum* and downregulated *Prevotella histicola*. In a small cohort study, Bankvall et al. (106) performed 16S rRNA gene sequencing and metaproteomics on buccal swab samples, identifying bacterial species and proteins with inflammatory and virulence potential upregulated in oral lichen planus patients.

The skin is the largest organ in our body, acting as the first line of defense against environmental changes, including pathogen infections. The skin microbiome plays a crucial role in modulating the immune states of both the skin locally and the entire human body immune system, contributing to resistance against pathogens and other environmental disturbances. It has been well-documented that the skin microbiome is implicated in diseases such as atopic dermatitis and psoriasis (107). As with other human microbiomes, most reported skin microbiome studies were performed with DNA sequencing. MS-based metabolomics approaches have also been frequently applied to study host–microbiome interactions on skin for skin diseases (108–110). To the best of our knowledge, no metaproteomic research articles on human skin microbiomes have been published so far.

4. ASSAYING THE MICROBIOME ACTIVITY FOR CLINICAL APPLICATION

A growing number of studies report prognostic traits/biomarkers within our microbiomes for diseases, as discussed in this review, and many others support the modulation of microbiome composition and function as viable therapeutic options. Metaproteomics, MS technologies, and bioinformatic software suites complement and build on these findings by directly exploring changes in microbiome pathways, functions, and metabolic outputs. The goal, then, of a functional microbiome assay is to utilize these prognostic traits, biomarkers, and specific microbial functional/metabolic profiles in live, individual, and representative microbiome cultures to develop treatments tailored to a particular health outcome or treatment response.

4.1. Live Microbiomes: Collection, Processing, and Storage

The collection, processing, and storage of live microbiome samples are critical first steps in the subsequent success and reproducibility of functional microbiome assays for clinical applications. Kennedy et al. (111) examined the challenges of studying low-biomass microbiomes. They highlighted that contamination was of concern and developed a strategy to remove contamination signals. Although they studied noncultivated samples, their results underscored the potential for contamination, which could be amplified during the live culture of low-biomass microbiomes. Groups should systematically test their microbiomes during collection and processing to exclude contamination. Recently, we detailed our standardized methods for fecal microbiome collection, processing, and storage and described how we assess these using a standardized functional assay (14). We also established transport and storage conditions for the human fecal microbiome, which showed that samples were stored more than a year at −80°C in a cryopreservation buffer

containing 10% glycerol while maintaining cultivability and functional activity (112). Burz et al. (113) provide a standardized protocol for collecting and storing stool samples intended for FMT, optimized for lyophilized samples and using a different cryopreservant. Thus, standardization must consider the end usage of the microbiome. Superdock et al. (114) compared processing and storage methods across oral and gut microbiomes, showing significant differences in community compositions, which, no doubt, would also be reflected in functional assay outputs. They caution against comparing results across different processing methods. Collection, processing, and storage standardization must be tailored to the microbiome of interest. Depending on the collection site, samples may require filtration or differential centrifugation during processing to remove human cells or other large debris. Both low-speed centrifugation and filtration have been employed to enrich gut microbiome from fecal debris (14). Low-biomass sources of microbiome samples may require centrifugation to concentrate microbial cells for later culture. Some microbiome samples, such as the fecal microbiome, require anaerobic collection, processing, and storage to preserve oxygen-sensitive microbes.

However, regardless of the location/source of microbiomes, several considerations are in common. (*a*) Samples should be processed as quickly as possible after collection to maintain viability; (*b*) standard aseptic techniques should be followed to prevent contamination; (*c*) protocols for a particular microbiome site should be optimized and standardized for consistency across studies; (*d*) during processing, microbiome samples should be homogenized to ensure a representative mix of the microbial community; (*e*) live microbiome samples should be divided in multiple aliquots as appropriate for downstream assays to prevent repeated freeze-thaw cycles and loss of viability; and (*f*) appropriate temperature control and addition of cryoprotectants to preserve microbial viability from collection to storage should be maintained.

4.2. Functional Microbiome Assays

Functional microbiome assays for clinical applications should (*a*) be high throughput to allow for the analyses of multiple samples efficiently, (*b*) provide sufficient output for insights into the functional activities of the microbiome, (*c*) facilitate personalized approaches to interventions based on individual microbiome functional profiles and changes, and (*d*) provide data in a timely manner for decision making.

Functional assays for the gut microbiome such as gut-on-a-chip (115–117), organoid cultures (118–120), SHIME (stimulator of the human intestinal microbiome ecosystem) models (121, 122), and RapidAIM (rapid assay of individual microbiomes) are well-established and amenable to metaproteomic and metabolic analyses (14, 123, 124) but with distinct considerations for clinical assay.

Gut-on-a-chip systems are microfluidic devices that integrate living human gut cells and microbiomes, allowing for real-time study of gut physiology and microbial interactions (115). These models allow the study of gut barrier function and permeability, validation of drug adsorption and transport, drug metabolism, and the effect of drugs on the microbiome, in addition to analysis of microbial-host interactions and response to drugs or diet. However, the small sample sizes in the microfluidics chamber limit the number of cells available for functional assays, and the low concentrations and volumes of metabolites produced can make measurements challenging. Although considered low throughput, recent advances in automation and multiplexed microfluidic platforms have increased the scalability of gut-on-a-chip. Early iterations were limited to aerobic culturing; however, the HuMiX (human microbial cross talk) model allows partitioned but proximal coculture of human and microbial cells under microaerophilic conditions (116). Recently, Lucchetti et al. (117) integrated thin-film electrodes into the HuMiX system to measure barrier tightness

and integrity, an important measure of the ability of substances to cross from the microbiome to the host.

Like gut-on-a-chip, the gut organoid culture offers the opportunity to study human gut cells and microbiome in coculture. These are representative two-dimensional (2D) and three-dimensional (3D) cultures of the human intestine derived from isolated crypts or stem cells. They replicate the cellular diversity and architecture of the gut, providing a realistic model for studying microbial interactions and gut biology (118). These systems allow one to study host–microbiome interactions and test the effects of drugs, probiotics, and other stimuli. However, they require the ability to generate and store patient-specific organoids for personalized studies, which can be time-consuming and invasive because they require a biopsy at the site. Despite these limitations, these techniques can be scalable for screening therapeutic compounds. Williamson et al. (119) developed a high-throughput organoid microinjection platform. Organoids (125) and organs-on-a-chip (126) are available for other sites of interest in microbiome research, such as oral (127, 128), lung (129), skin (130) and vaginal (131) sites. Limitations for these sites include low microbiome biomass, difficulty with reproducible cultures, and the limitations mentioned above.

SHIME models are multichamber systems designed to mimic the conditions of the entire gastrointestinal tract, including the stomach, small intestine, and ascending, transverse, and descending colons (121). Not originally designed as high-throughput systems, they have undergone several iterations to simultaneously allow for testing multiple conditions. For instance, the QuadSHIME tests four conditions in a single setup (122). SHIME models enable long-term and repeated dosing over four weeks. They are reproducible, generating ample materials for multi-omics studies and thus facilitating comprehensive studies of microbiome interactions, functions, and responses to various conditions. Despite these advantages, these systems are large and require more time and resources, which are important considerations when scaling for larger populations or routine clinical studies.

RapidAIM is an innovative approach designed for high-throughput assessment of the functional potential of individual microbiomes in a 96-well plate format. It can readily assess the effect of drugs, prebiotics, and other compounds on human gut microbiomes (14, 123, 124). By using metaproteomics, we have demonstrated that RapidAIM maintains the compositional and functional profile of the microbiome and replicated the in vivo effects of drugs, such as metformin (123). Recently, Zund et al. (132) introduced a 96-well high-throughput assay for the culture of gut microbiome that also replicated in vivo drug effects. These assays can be standardized across laboratories and are time- and cost-effective. They provide sufficient material for downstream omics or targeted analyses. Although these assays do not offer coculture with human gut tissue, they are scalable for large population studies and routine clinical use. RapidAIM runs typically take 18–24 h but can extend to five days (123).

Each assay system offers unique strengths and insights, and when combined, they provide a holistic view that is crucial for clinical applications and research. Ideally, a fast-pass functional assay such as RapidAIM could be designed for other microbiome sites, generating a functional outlook to a stimulus/condition. Then, if required, a more in-depth assay such as the organoid or organ-on-a-chip could be applied with the end goal to facilitate evaluation of microbiome-targeted therapies and predict an individual's drug responses. These assays can be used to assess how the microbiome affects the drug metabolism, or conversely, how the microbiome is affected by the drug, which ultimately inform stratification of patients to tailor treatments accordingly. Disease progression and response to treatment can be monitored by regular interval collection and analysis of patient samples integrated with functional meta-omic outputs.

4.3. Drug Screening, Reclassification, and Repurposing

The field of drug screening, reclassification, and repurposing in the context of microbiome research is a rapidly evolving area. The core idea is that many drugs can significantly affect the human microbiome, and conversely, the microbiome can affect drug availability and action (133). These effects may contribute to the drug's therapeutic action, influence its side effects, or alter its metabolism. The individualized response to drugs has been recognized for decades, causing delays in effective treatments and potential for adverse effects. A classic example is digoxin, used for treating heart failure (134). Digoxin can be converted to an inactive metabolite by a glycosidase reductase found in some strains of *Eggerthella lenta* for 10% of patients, limiting the availability of the active drug for absorption and its therapeutic usage (135).

Drug screening using functional microbiome assays coupled with meta-omic outputs can identify existing drugs that interact with the microbiome, identifying those compounds that modulate the microbiome and vice versa, uncovering drugs that work through microbiome-mediated mechanisms, and be utilized as a screening tool for potential adverse effects of drugs on the microbiome. Some work has been done using individual bacterial strains. For instance, Zimmermann et al. (136) examined the ability of 76 human gut bacteria to metabolize 271 oral drugs, finding that 176 were significantly reduced by at least one bacterial strain using LC-MS. Wang et al. (137) have developed an activity-based screening platform from human fecal samples. They screened for the activity of 110 selected human enzymes important in disease or that are targets of drug metabolism, showing that 71 were present as isoenzymes in fecal samples. Focusing on dipeptidyl peptidase 4 (DDP4), an enzyme that metabolizes glucagon-like peptide 1 (GLP1) to an inactive form, they reported microbiome-derived DDP4 contributed significantly to GLP1 inactivation and was, in fact, resistant to inhibitors, such as sitagliptin, designed against human DDP4 (137). We utilized our 96-well assay, RapidAIM, together with quantitative metaproteomics to demonstrate its utility in quantifying the effect of 43 drugs on five individual microbiomes. The biomass of at least one species was altered by 35/39 nonantibiotic drugs, with 535 clusters of orthologous groups (COGs) significantly decreased by at least one drug, and functional changes in enzyme pathways were noted (124). Interpersonal variability can be assessed by using individual microbiomes over pooled samples, moving the field toward personalized/precision medicine.

Reclassification of existing drugs based on their effects on the microbiome is an emerging field reshaping our understanding of drug action and efficacy. It can lead to a better understanding of drug mechanisms, improved drug efficacy and side effect prediction, and new classification systems that account for microbiome impacts. Maier et al. (138) screened 1,000 nonantibiotic drugs against 40 gut bacterial strains to find that 24% impacted the growth of at least one bacterial strain, suggesting a need to reclassify drugs based on their microbiome effects. Metformin, used to treat type 2 diabetes, reduces glucose production in the liver; however, several studies noted it caused large changes in the gut microbiome, contributing to increased SCFA production and restrained insulin secretion in the adipose tissue (139).

In the future, more systematic, high-throughput assays should be employed to test large libraries of existing drugs against diverse bacterial strains or complex microbial communities from representative individuals. Following drug exposure, microbial metabolites and affected pathways, proteins, and taxa can be quantified via MS-based functional omics approaches, such as metaproteomics and metabolomics. Reclassification could lead to more personalized drug prescriptions based on an individual's microbiome profile. Understanding a drug's effect on the microbiome might reveal new potential uses for existing drugs: drug repurposing. Benefits include faster and cheaper drug development processes and the potential for new treatments for conditions with unmet medical needs. In the future, we need to consider microbiome effects in drug development

pipelines, integrating microbiome data into drug databases, thereby recognizing the potential for new drug classes specifically targeting microbiome modulation.

5. CONCLUDING REMARKS

Recent technological advancements enabled metaproteomics to study various types of human microbiomes (e.g., fecal, mucosal, airway, oral, and urogenital) under different health and disease conditions. Compared to the commonly used metagenomic sequencing methods, metaproteomics offers significant advantages that align well with the evolving focus of microbiome research, namely a shift from taxa or gene cataloging to more functional profiling over the past several years. Looking ahead, clinical and translational microbiome research will benefit from the application of functional microbiome assays based on live microbiome biobanking and rapid testing, which can inform disease treatment and patient stratification and facilitate drug development. MS-based functional meta-omics, including metaproteomics and metabolomics, will play crucial roles in these areas due to their unique capability to directly measure expressed microbial enzymes, protein PTMs, and metabolic outcomes (**Figure 3**).

Technically, MS-based metaproteomics has significantly progressed over the past five to ten years, with the development of more user-friendly software tools and higher-performance mass spectrometers. These advancements dramatically increased the depth of measurement, from around 1,000 microbial proteins to >50,000 proteins identified in a single microbiome study. Although this depth of measurement still represents only a small portion of the theoretically present proteins in the human microbiome (27), a large amount of functional, pathway, and taxonomic information can be derived from metaproteomics data for better understanding of the host–microbiome interactions. It is important to note that the field is rapidly evolving, continually

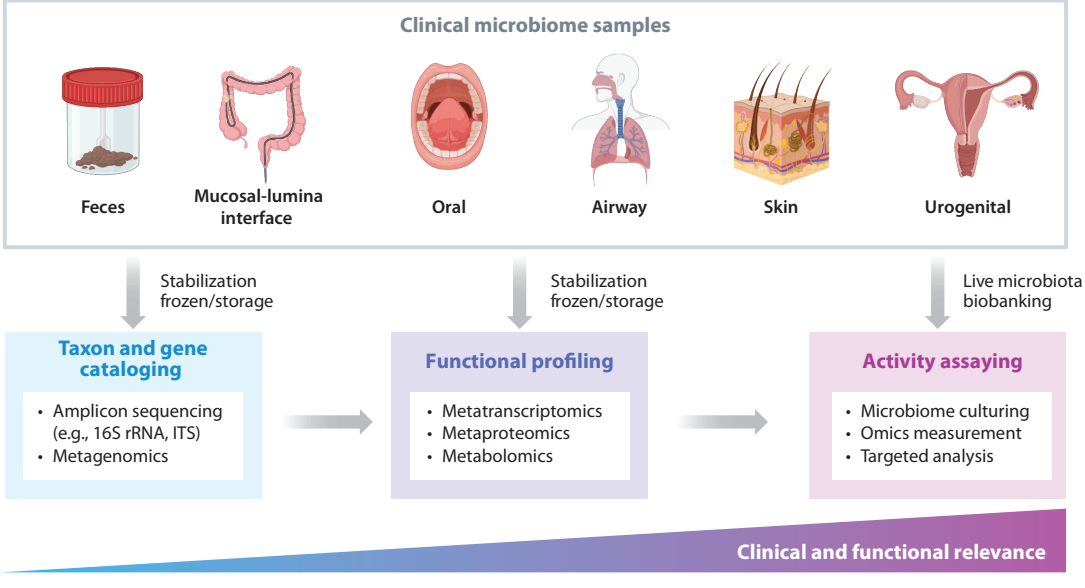

Figure 3

Clinical microbiome sample analysis with meta-omics and functional microbiome assays. The microbiome research is evolving with a need for different bioanalytical methods at different stages. Functional approaches, such as metaproteomics, metabolomics, and live microbiome assays, are needed for more functional and translational investigations. Abbreviations: ITS, internal transcribed spacer; rRNA, ribosomal RNA. Figure adapted from images created with BioRender.com.

pushing the boundaries of metaproteomic identifications in microbiome. Despite these promising developments, we believe that the metaproteomics field could benefit significantly from additional efforts in the following areas.

1. New strategies and technologies. There is still a need to develop and apply new technologies for metaproteomics, including bioinformatics tools, to fully exploit the benefits of DIA metaproteomics. Another important aspect is the adaptation of de novo methods (140). This approach can generate a pseudomatched FASTA database without prior knowledge of microbial composition or metagenomic data, which is often the case for many underinvestigated human microbiomes. It also allows for the discovery and profiling of unknown functional peptides and proteins in microbiome samples. Metaproteomics often adopts technologies and strategies developed for conventional proteomics. However, metaproteomes have unique characteristics that require additional consideration and evaluation. For example, the match-between-runs function needs to be used with extreme caution in DDA metaproteomics. The default MS1-level filtering threshold, which includes *m/z*, charge state, retention time, and ion mobility when applicable, is often insufficient for transferring identifications from one sample to another due to the overwhelming complexity (141), particularly in metaproteomics.
2. Large-scale flagship clinical metaproteomics studies. To date, there is still a lack of clinical metaproteomic studies with sufficient sample sizes, comprehensiveness of data analysis, and clinically impactful biological discoveries to sufficiently showcase the unique capabilities of metaproteomics. The application of microflow LC DIA-MS and automated sample preparation workflows could help achieve this goal. Additionally, integrated multi-omics analysis, which includes metaproteomics or metaPTMomics (76) as well as commonly used metagenomics or metatranscriptomics (74), can aid in the demonstration of the unique and added insights by metaproteomics for better understanding the roles of microbiome in diseases.
3. Standardization and quality control. Standardization and efficient quality control are crucial for the application of metaproteomics in clinical sample analysis. While the standardization of experimental workflows, including bioinformatics analysis, may not always be possible or reasonable from an innovation standpoint, establishing proper data reporting guidelines is essential to ensure data quality and traceability (142). Either in-house quality control samples or external microbiome reference reagents are needed at every step of the workflow, including sample preparation and MS data acquisition. In proteomics, it is widely agreed that all MS raw data must be deposited in public repositories and made available with sufficient metadata. The Sample and Data Relationship Format (SDRF) provides good examples of the necessary information (143, 144). For clinical applications, including additional metadata such as age and dietary information is encouraged.

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