Microbial Metabolomics: From Methods to Translational Applications

Rui Guo, Xialin Luo, Xu Xin, Lian Liu, Xijun Wang, and Haitao Lu

1 Introduction

Microbes, which are generally divided into five categories, namely, bacteria, viruses, fungi, archaea, and protozoa, are the pathogens responsible for many infectious diseases that remain a leading cause of death worldwide due to the ongoing emergence of new pathogens and resurgence of previous pathogens. For instance, the gut microbiota is a contributing factor to the pathophysiology of obesity. *Escherichia coli* is the major pathogen of urinary tract infection (UTI), which has a high rate of recurrence. Hepatitis C virus (HCV) induces the development of hepatitis C, and some other serious infectious diseases, even cancer, are associated with microbes [1–4].

Conventionally, molecular and cellular biological methods are used to study infections at the gene and protein levels, but these methods cannot be used for precise and direct monitoring of minor changes in biological niches, let alone for pathogenic annotation. As the final downstream event of transcription and translation, metabolism will amplify these changes, which can then be traced back to easily identify the pathogenesis of infectious diseases [5]. To our knowledge, microbial metabolism refers to a series of chemical reactions that occur during the growth, proliferation, and differentiation of microbes and substrate degradation by microbes, including catabolism and anabolism, which are closely associated with pathogenesis and virulence [6]. Therefore, microbial metabolomics provide a new opportunity to study the diagnosis, pathogenesis, and treatment of diverse infections.

Over the past few years, microbial metabolomics, which is designated for global profiling of a large number of small molecules (molecular weight <1000) from a microbiological system,

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has developed rapidly and been introduced into life science research. Owing to the development of techniques such as high-resolution mass spectrometry (MS), methods of sample preparation and biological annotation databases such as the Human Metabolome Database (HMDB), we can precisely target microbiological functions and then apply microbial metabolomics to study the diagnosis, pathogenesis, and treatment of many infections, such as type 1 diabetes (T1D), UTI, and cystic fibrosis (CF) [2].

In this chapter, we attempt to summarize microbial metabolomics from methods to applications in the study of diverse infectious diseases, allowing us to better understand how microbial metabolomics aids the study of the diagnosis, pathogenesis, and treatment of microbe-related infections (Fig. 1).

2 Methods in Microbial Metabolomics

2.1 Key Analytical Tools for Microbial Metabolomics

Microbial metabolomics strives to analyze the metabolomes of microbiological systems and then translates the metabolic differences to phenotypic differences, enhancing our knowledge about the molecular mechanisms of infectious diseases [7]. Accuracy, sensitivity, and high throughput are three basic characteristics that analytical instruments used for microbial metabolomics should possess. However, because the classes and concentrations of metabolites vary greatly and due to the presence of a large number of metabolites in biological samples, it is very challenging for only one analytical tool to meet all three requirements and detect all known and unknown metabolites [8]. Two types of instrumentation platforms based on nuclear magnetic resonance (NMR) and MS are currently preferred, although neither of these platforms can profile all the metabolites present in microbial samples in an unbiased manner.

NMR spectroscopy identifies chemical structures based on the absorption spectra of radio frequency (RF) pulses from the nuclei of atoms in strong magnetic fields. The commonly employed atoms for analysis include ^{1}H, ^{13}C, and ^{31}P, and this technique can analyze metabolites in samples both qualitatively and quantitatively at the same time. The applications of NMR spectroscopy in metabolomics vary widely, with the most common application being qualitative and quantitative analysis of metabolites in body fluid samples. For example, ^{1}H-NMR was applied to

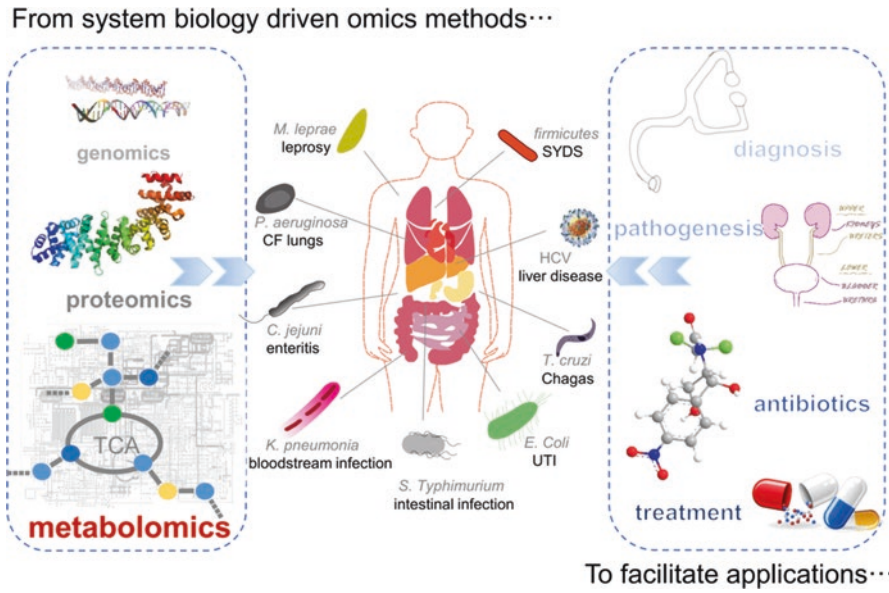

Fig. 1 Applications of metabolomics in the study of microbe-related infectious diseases

compare metabolomes between biofilms, which are associated with many infections, and planktonic cells of *Staphylococcus aureus* [9]. However, due to the complexity of metabolites from microbes and wide range of metabolite concentrations, as well as the relatively low sensitivity and high cost of NMR, the application of NMR in microbial metabolomics has been limited.

To overcome these problems, high-resolution MS has recently become an indispensable tool in metabolomics. Sample preparation techniques such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) coupled with high-resolution MS methods are broadly employed in microbial metabolomics [7].

GC-MS is a hyphenated technique in which the metabolites of the sample are first separated by GC and then detected and identified by MS [10]. GC-MS only detects volatile and thermally stable compounds, while a majority of microbial metabolites are nonvolatile and thermally labile, such as phosphorylated metabolites, which may degrade when placed at high temperatures in the GC oven. However, this method has been used to analyze microbial metabolites after derivatization due to the advantages of GC, including the ability to efficiently distinguish isomeric compounds, the ease of use, and the low cost compared to other separation tools [8]. For example, a derivatization reaction combined with GC-MS analysis has been used to study numerous microbes, such as *Propionibacterium freudenreichii*, *E. coli*, and *Bacillus subtilis* [7].

LC-MS is another hyphenated technique that offers analyte separation via LC followed by ionization and MS detection. There are two commonly used ionization methods for LC-MS, namely, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), which are both very sensitive. However, ESI is more desirable in microbial metabolomics because ESI-MS preferentially detects polar compounds, while APCI-MS detects nonpolar compounds. In addition, in contrast to GC-MS, LC-MS does not require high temperatures and volatility of analytes, which increases the ease of sample preparation [10]. Furthermore, LC-MS is also highly sensitive with small sample volumes [11]. For example, myxoprincomide, a novel myxobacterial metabolite of *Myxococcus xanthus* DK1622, was discovered by LC coupled with high-resolution MS (LC-HRMS) [12]. Nevertheless, there remain some challenges associated with LC-MS techniques, such as interference by the high salt content in microbial media samples, ionization suppression, and the relatively low resolution of high-performance LC (HPLC). Nevertheless, the emergence of ultrahigh-performance LC (UPLC) has improved chromatographic resolution greatly [10]. For instance, Marcobal and colleagues used an UPLC-MS method to examine the influence of the gut microbiota on the urinary and fecal metabolome of a humanized mouse [13]. CE-MS is another useful tool for metabolomic analysis, the advantages of which include exquisite separation efficiency, very small sample volumes (nL range), and low cost when compared to GC-MS and LC-MS. However, the major shortcoming of CE-MS is the difficulties at the interface between CE and MS [11].

In short, there are several platforms employed in microbial metabolomics (Fig. 2), and each type of instrument has advantages and disadvantages. Researchers should choose the best method or combine the tools to analyze microbial metabolites based on the characteristics of compounds of interest and analytical tools (Fig. 2).

2.2 Sample Preparation and Data Mining of Microbial Metabolomics

Microbial metabolomics focuses on intracellular metabolites that change quickly over time. Therefore, the methods and conditions of sampling and sample preparation, including time, storage condition, and other factors, greatly influence the reproducibility, precision, and accuracy of detection. In addition, the biological variability tends to be larger than analytical variability, which enhances the importance of optimizing sampling and sample preparation methods.

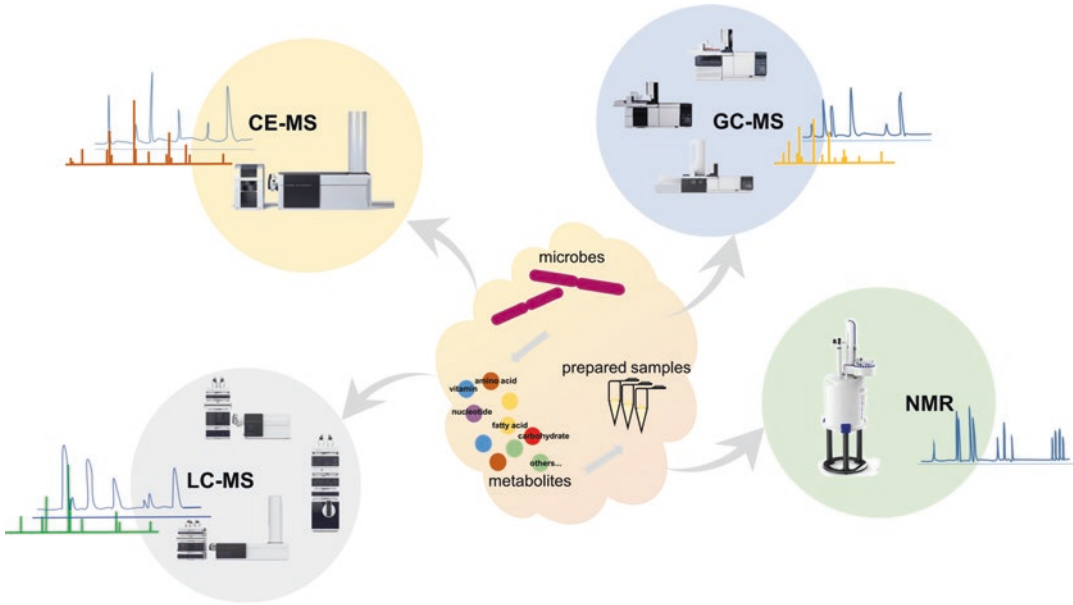

Fig. 2 Platforms used for microbial metabolomics

Some metabolic processes are so rapid (usually less than 2 s) that quick collection of samples from the reactor to stop cell metabolism, especially enzymatic processes, is very crucial. Quick harvesting of metabolites followed by freezing in liquid nitrogen and then storing at −80 °C is commonly used by many researchers. Choosing an effective approach for instant quenching is also rather important for the harvesting of metabolites, and the approach should meet basic requirements such as absence of cell leakage or detection of any leaked metabolites. The use of acidic reagents such as nitric and perchloric acid drastically reduces the number of detected metabolites, and unstable compounds are severely degraded. Hot alcoholic polar (e.g., methanol/water) and nonpolar (e.g., chloroform) extractions are also employed. According to many related studies, prokaryotic microbes such as *E. coli* have a greater tendency to exhibit leakage of intracellular metabolites than eukaryotic microbes such as yeasts when treated with cold methanol, which might be due to the differences in cell wall and membrane structures between the two types of microorganisms. Therefore, cold methanol extraction may be promoted as a common quenching method for extraction of intracellular metabolites from some prokaryotic microbes [14]. A detailed procedure for sampling and sample processing is shown in Fig. 3.

After analyzing the samples via MS-based platform, we obtained the raw data that were very complex. Therefore, data analysis requires the use of appropriate informatics tools for metabolite identification and quantification (Fig. 3). First, pretreatment of raw data to exclude irrelative factors is important and indispensable. Several major processes that involve noise filtering, resolution of overlapping peaks, peak alignment, peak matching, and peak normalization are needed. Current software programs for performing data pretreatment include MetAlign, MET-IDEA, MZmine, Progenesis QI, XCMS, and MSFACTs. The first three can be used to pretreat all LC-MS and GC-MS raw data, while Progenesis QI and XCMS can only process data produced by LC-MS, and MSFACTs is a software for GC-MS data processing. Among the tools mentioned above, MetAlign and MZmine cannot resolve overlapping peaks; MET-IDEA can extract semiquantitative information in addition to resolving overlapping peaks; and XCMS can perform alignment of nonlinear retention times, noise filtering, and other functions. STOCSY (statistical total correlation spectroscopy) is a commonly used method for molecule

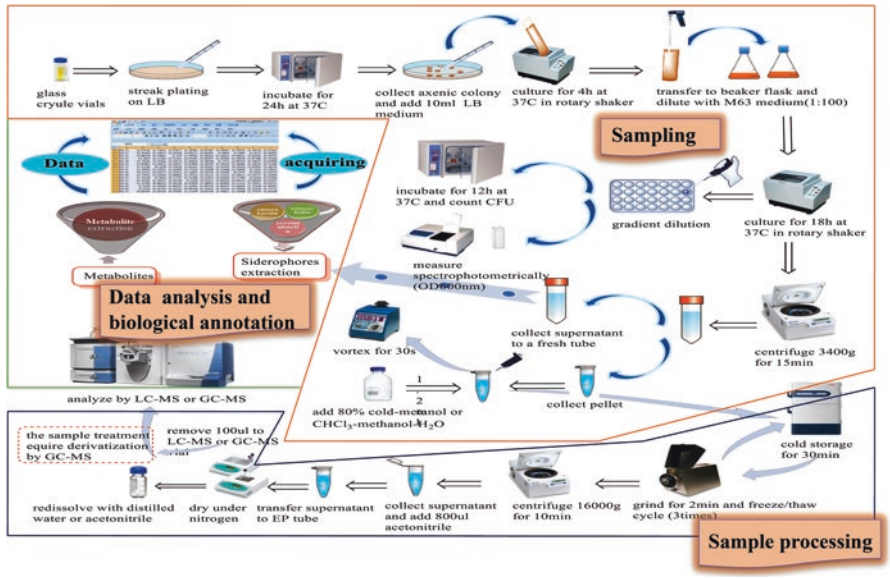

Fig. 3 Sample preparation and data mining of metabolites from *E. coli*

identification in microbial metabolomics based on NMR. It takes advantage of the multicollinearity of the intensity variables in a set of spectra to generate a pseudo-two-dimensional NMR spectrum that displays the correlation among the intensities of the various peaks across the whole sample. This method is not limited to the usual connectivities that are deducible from more standard two-dimensional NMR spectroscopic methods, such as TOCSY. Moreover, two or more molecules involved in the same pathway can also present high intermolecular correlations because of biological covariance or can even be anticorrelated. The combination of STOCSY with supervised pattern recognition and particularly orthogonal projection on latent structure-discriminant analysis (O-PLS-DA) offers a new powerful framework for analysis of metabolomic data. In a first step O-PLS-DA extracts the part of NMR spectra related to discrimination. This information is then cross-combined with the STOCSY results to help identify the molecules responsible for the metabolic variation [15].

In addition to data pretreatment, mining of useful information from a large amount of data and functional annotation of this data is another key challenge in microbial metabolomics. Multivariable data analysis (MVDA) is commonly used to extract information from data sets, including analysis of variables that contribute to classification, identification of biomarkers associated with phenotypes, and annotation of regulatory mechanisms via metabolic pathways. Recently developed MVDA techniques comprise supervised and unsupervised methods as two main types. In microbial metabolomics, supervised methods include clustering analysis (CA) and principal component analysis (PCA), and unsupervised methods include linear discriminant analysis (LDA), partial least squares (PLS) analysis, partial least squares-discriminant analysis (PLS-DA), and artificial neural network (ANN) analysis. Among these analytical methods, PCA and PLS-DA are the most commonly applied methods, yielding classification information via a score plot and revealing metabolites that contribute to the classification as well as the determining the contribution of these metabolites via a loading plot [3, 16, 17].

2.3 Biological Annotation of Differential Metabolic Pathways Characterized by Microbial Metabolomics

After identification of the contributive molecules, the next important step is to identify relevant metabolic pathways that could explain the roles of these molecules in metabolism. Identification

of metabolic pathway has actually helped elucidate the connections among metabolites and proven to be effective for understanding pathway genes/enzymes and related molecular biology [18]. Over the past decade, many excellent online metabolic pathway databases have emerged to provide intuitive bioinformatic tools for the visualization, interpretation, and analysis of pathways (Fig. 4), such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/), BioCyc (https://biocyc.org/), Reactome (https://reactome.org/), Small Molecule Pathway Database (SMPDB) (http://www.smpdb.ca/), and Metabolomics Pathway Analysis (MetPA) (http://metpa.metabolomics.ca/) [18–24]. The KEGG pathway database is a reference database consisting of metabolic pathway maps with functional significance [25]. BioCyc provides not only a reference for genomes and metabolic pathways but is also a powerful computational analytical tool for prediction of metabolic pathways and operons; BioCyc includes EcoCyc, which is a specific database for the bacterium *E. coli* K-12 MG1655. The Reactome database is based on reactions that are grouped into causal chains to form pathways in human systems [20].

Metabolic pathway analysis is usually based on high-throughput metabolomic data achieved by NMR- or MS-based analysis of biological samples. Correct mapping of metabolic pathways relies on robust data processing and analysis, such as identification and characterization of metabolites, and visualization of the results. In targeted metabolomics (quantitative metabolomics), compound identification and quantification are usually achieved by comparing analytical samples on the basis of a series of chemical standards [26]. For statistical analysis of targeted metabolomic data, openly accessible software/database tools such as MetaboAnalyst (http://www.metaboanalyst.ca/), MeltDB (https://meltdb.cebitec.uni-bielefeld.de/), HMDB, and MeTPA are usually preferred [26–28]. In

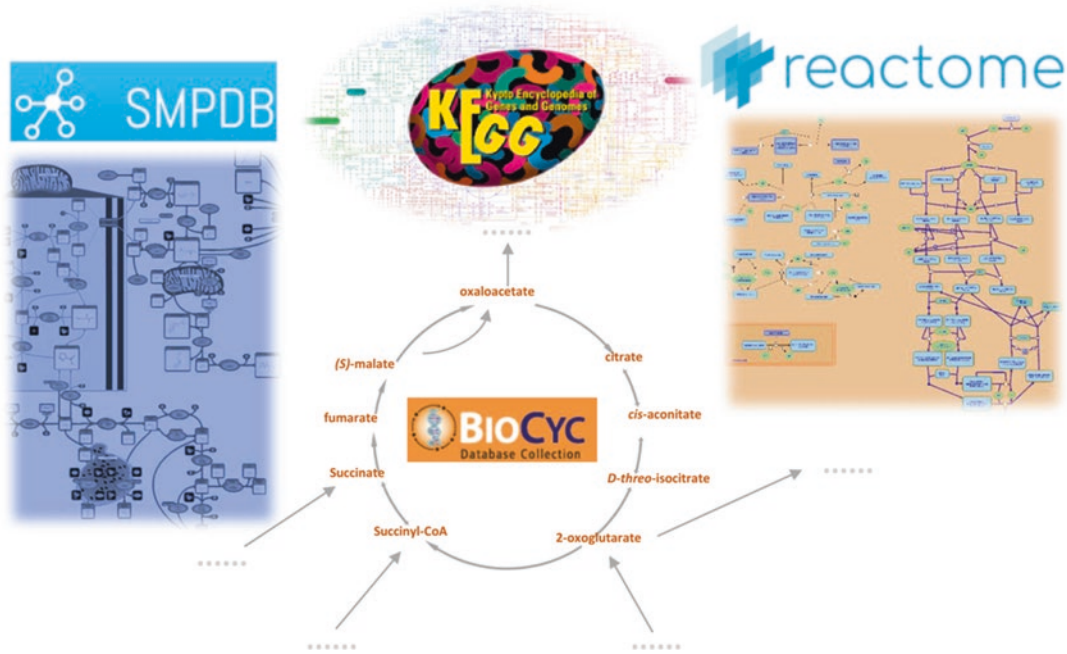

Fig. 4 Online metabolic pathway databases for biological annotation

MetaboAnalyst, detailed and hyperlinked diagrams of pathways can be obtained after uploading a peak list and including information regarding the name and peak intensity of each identified metabolite. MeltDB is similar to MetaboAnalyst to some extent but is only used for MS-based metabolomics data analysis. All the results are linked to the HMDB, which is currently the most comprehensive database of human metabolites and related metabolisms [29]. MetPA is another powerful tool that helps identify the most relevant metabolic pathways and visualizes pathway data.

Metabolic pathway analysis of untargeted metabolomic data is quite challenging because it requires confident identification of a large number of metabolites and building of complex relationships among the identified metabolites. Therefore, comprehensive databases such as the HMDB, METLIN (https://metlin.scripps.edu/), and Madison Metabolomics Consortium Database (MMCD) (http://mmcd.nmrfam.wisc.edu/) [29, 30] are needed to provide the structural properties (e.g., MS/MS spectra) and functional properties of metabolites. Such publicly accessible, web-based databases provide hyperlinks to other databases and share some information in common. HMDB integrates information regarding compound description, chemical structure, and disease associations and reference NMR and MS spectra [29]. METLIN is centered on MS-based data, especially MS/MS data. This database can be used to match metabolites based on MS and MS/MS data. Recently, isoMETLIN, a version for isotope-labeled compounds, has facilitated untargeted global isotope-tracer experiments [30]. The MMCD database supports an extensive search using experimental MS or NMR data, and this database contains information for more than 20,000 biologically relevant small molecules chosen from KEGG, BioCyc, HMDB, and others [31]. These open-source databases allow their content and software infrastructures to be optimized and updated according to user feedback, greatly increasing the convenience and efficiency of metabolic pathway analysis of biological systems.

3 Translational Applications of Microbial Metabolomics

3.1 Diagnosis of Infectious Diseases Caused by Pathogenic Microbes

Many infectious diseases, such as UTI, spleen-yang-deficiency syndrome (SYDS), and CF-associated lung disease, cause serious issues to patients' health. However, diagnosis of these diseases remains highly challenging due to the lack of effective biomarkers. Fortunately, the applications of microbial metabolomics have significantly contributed to the diagnosis of infection diseases. UTI is a serious disease worldwide that mainly affects females, and the most common pathogen is *E. coli* [2]. LC-MS was used to globally profile the metabolites in urine from healthy controls and patients with UTI, and several potential biomarkers were identified successfully (Fig. 5) [32]. Lam et al. applied proton NMR spectroscopy to analyzing 88 urine samples from UTI patients and demonstrated that trimethylamine (TMA) could serve as a human-microbial marker of UTI associated with *E. coli*, and NMR-based urinalysis could aid the etiological diagnosis of this infectious disease [33].

SYDS is a typical syndrome in traditional Chinese medicine (TCM). Patients with SYDS can be distinguished from healthy controls by performing liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QTOF-MS)-based metabolomics and 16S rRNA sequencing because the number of *Firmicutes* and *Clostridia* bacteria that contribute to energy dysfunction is increased in the gut of SYDS patients. Therefore, *Firmicutes* and *Clostridia* bacteria may become new markers for the diagnosis of SYDS via microbial metabolomics coupled with 16S rRNA sequencing [34]. *Pseudomonas aeruginosa* is one of the most common pathogens and causes CF-associated lung disease. By analyzing the metabolites of CF patients and non-CF patients via LC-MS/MS, sphingolipids were found to be the most abundant molecules in the sputum of CF patients. This study showed that microbial

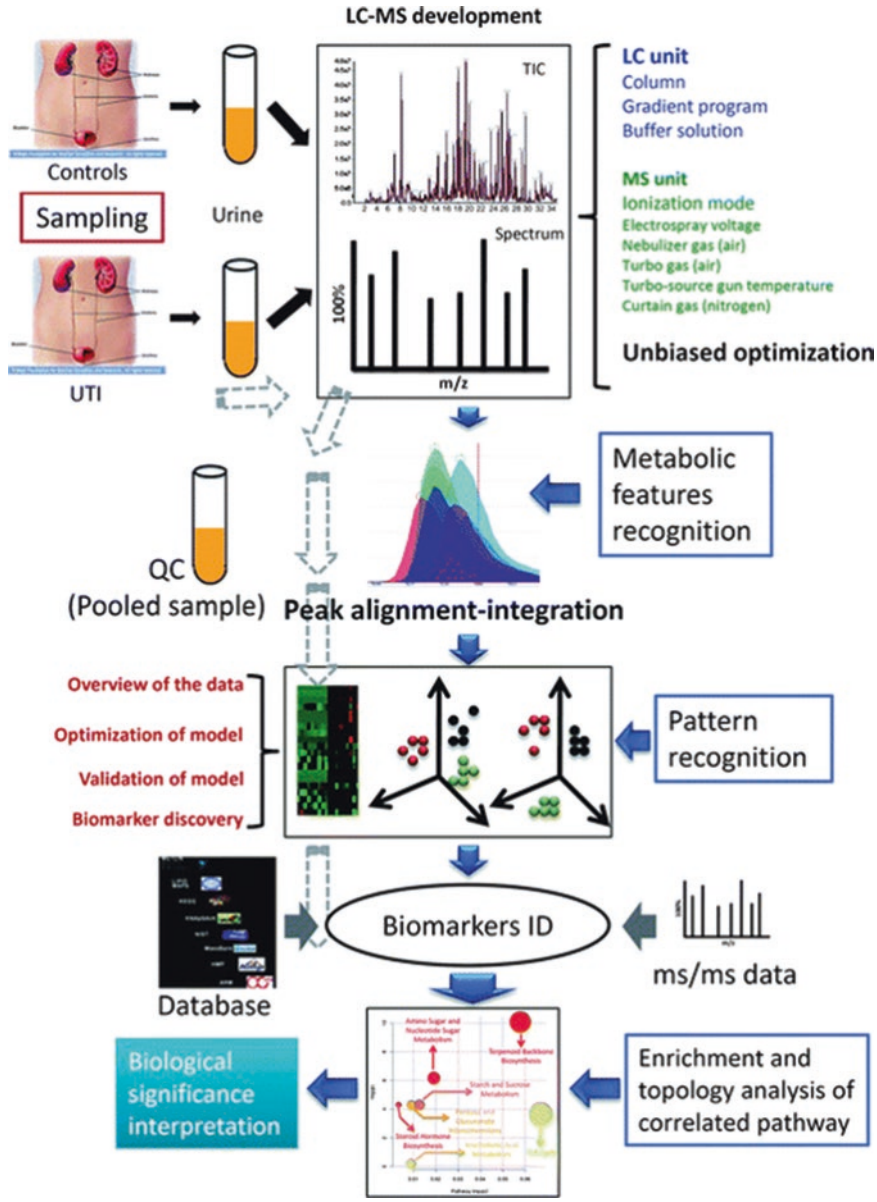

Fig. 5 LC-MS-based global metabolic profiling platform for human urine from healthy controls and patients with UTI

metabolomics could identify specific compounds that are abundant in clinical samples to help diagnose the disease [35].

Microbial metabolomics could also improve the diagnosis of inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC) caused by the gut microbiota by identifying the altered metabolite signatures in biological samples [36]. For instance, GC-MS was performed to analyze the fecal samples from 20 UC patients, 22 CD patients, 26 IBS patients, and 19 healthy controls and reveal the increased levels of ester and alcohol derivatives of short chain fatty acids (SCFAs) and indole in the CD group [27]. Based on ^{1}H-NMR analysis, the levels of 3-hydroxybutyrate, β-glucose, α-glucose, and phenylalanine were found to be significantly increased and lipid levels were significantly decreased in the serum samples of UC patients compared to healthy controls [4]. In addition, uri-

nary metabolites, including those originating from the gut microbiota, such as hippurate, acetate, methanol, methylamine and formate, TCA cycle intermediates, creatine, urea, taurine, and trigonelline, were identified as potential biomarkers to distinguish IBD patients from healthy controls [38]. Ahmed et al. reported a comprehensive study of the fecal volatile organic metabolites (VOMs) in the patients with diarrhea-predominant IBS (IBS-D, n = 30), CD (n = 62) or UC (n = 48), and healthy controls (n = 109). Fecal VOMs were extracted by solid-phase microextraction and analyzed by GC-MS. In total, 240 VOMs were identified. Esters of short chain fatty acids, cyclohexanecarboxylic acid, and its ester derivatives were associated with IBS-D, while aldehydes were more abundant in IBD. A predictive model, developed by multivariate analysis, could differentiate IBS-D from active CD, UC, and healthy controls with high sensitivity and specificity [39].

3.2 Pathogenesis Annotation of Microbial Infections

Although many drugs have been developed to treat infections, patients also experience side effects due to the nonspecificity of drug targets. In this regard, microbial metabolomics may be a useful tool to better understand the pathogenesis of infectious diseases and to promote precision treatment. Infections such as T1D, neonatal necrotizing enterocolitis (NEC), and UTI are increasingly prevalent conditions associated with gut microbiota, and the early mechanism of these illnesses remains elusive. By profiling the serum metabolites from transgenic mice with a combined LC-MS and GC-MS approach, decreased levels of lysophosphatidylcholine (LPC) and methionine and accumulation of ceramides were observed, which may facilitate our understanding of early T1D pathogenesis [40]. NEC mainly leads to the mortality of infants with very low birth weight. Metabolomics and next-generation sequencing tools have been used to investigate the contribution of intestinal microbes to NEC pathogenesis. The role of intestinal microbes was redefined and numerous evidences support the supported the hypothesis that NEC is a microbe-mediated disorder [41].

In addition, NMR has been used to determine the relationship between siderophores (secondary metabolites of microorganisms) and the pathogenesis of *E. coli*, the gut microorganism that causes UTI, demonstrating that the molecular interactions between the host and pathogen provide novel insight into pathogenesis (Fig. 6)

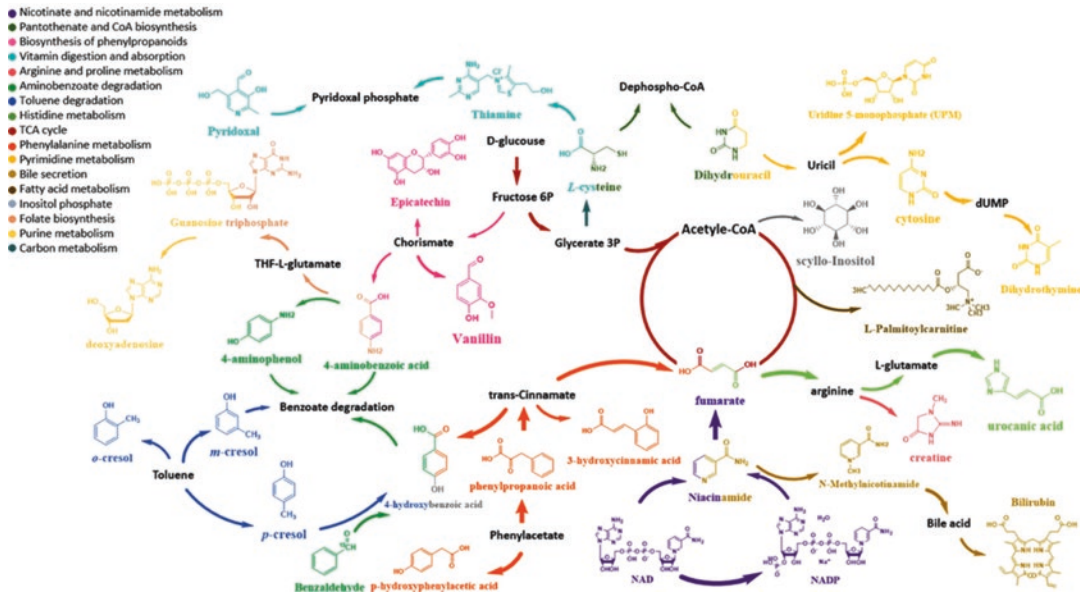

Fig. 6 Urinary metabolites altered by siderophore treatment, as identified via NMR spectroscopy, and related metabolic pathways

[42]. Similarly, GC-MS was employed to explore the relationships among the host, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), and the commensal gut bacteria during *S.* Typhimurium-mediated intestinal infection, and the data demonstrated the accumulation of metabolites consumed by commensal microbes. This result offers insights into the molecular interplay among the host, pathogen, and commensal microbes during pathogenesis [43].

Liver disease is a serious illness worldwide, and HCV is the leading cause, but the pathogenesis of HCV is not fully understood. Fortunately, some progress has been made with the help of microbial metabolomics. For instance, Sun and colleagues applied UPLC/ESI-SYNAPT-HDMS to analysis of metabolites of an HCV animal model and identified 38 distinct compounds, such as hypotaurine, glycerophospholipid, and tryptophan, as effective biomarkers for HCV diagnosis and pathogenesis [44]. Another microbe, *Mycobacterium leprae*, causes leprosy, which mainly infects the skin and peripheral nervous system. UPLC-MS was used to investigate the serum samples from the patients with high bacterial indices (BIs) and low BIs, and the levels of arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid were found to significantly increase, particularly in high-BI patients, which may serve as potential biomarkers and facilitate the study of high-BI pathogenesis [45].

3.3 Development of Antibiotic Resistance Against Microbe-Associated Infections

Penicillin and sulfonamide were the first two effective antimicrobials, and the former has saved thousands of lives. A number of other prevalent antibiotics such as streptomycin, aureomycin, chloramphenicol, and kanamycin were subsequently discovered. However, antibiotic resistance (AR) has emerged with misuse, overuse, and even underuse of antibiotics. In fact, the main reason for the lack of success in AR control is that the wide range of biochemical and physiological mechanisms is poorly understood due to the complexity of the processes that contribute to the emergence and dissemination of resistance [46]. Nonetheless, microbial metabolomics may have potential applications in the control of AR because most AR processes consume cellular energy, which leads to clear downstream changes in microbial metabolism [47].

Biofilms are sessile communities of microbes, usually bacteria or fungi, on surfaces or liquid-air interfaces. Biofilms are closely associated with many health problems, such as UTI, dental caries, chronic osteomyelitis, and CF-associated lung infection. However, these diseases are difficult to treat due to the resistance of biofilms to antibiotics [2]. To overcome the resistance, both MS- and NMR-based metabolomics have recently been used to study biofilms. For instance, Stipetic et al. demonstrated a novel extraction method via bead beating in a chloroform/methanol/water extraction solvent, and the metabolites were then analyzed by LC-MS to detect metabolic alterations between biofilm and planktonic cells of *S. aureus*. Significant changes in arginine biosynthesis were identified [48]. Another study by Hess et al. was performed on a biofilm of *S. aureus* with ^{1}H NMR, and low oxygen concentrations were found to inhibit biofilm formation and regulate the ability of gentamicin and vancomycin. The results showed differential metabolomic profiles between aerobic and anaerobic biofilms and demonstrated that microbial metabolomics is an effective tool for identification of the main molecules involved in biofilm development [49]. In addition, mannoside was shown to potentiate the activity of trimethoprim-sulfamethoxazole in the treatment of UTI [50]. All these studies indicate that microbial metabolomics may serve as a powerful tool to understand the mechanisms underlying the resistance of biofilms to antibiotics.

An untargeted metabolomics approach was used to quantify the short-term metabolic changes that occur in treating *E. coli* with several antibiotics; this study was performed with QTOF-MS to understand the mechanisms of drug action and determine approaches to potentially address AR (Fig. 7). The results revealed that an imbalance of ammonium could improve chloramphenicol tox-

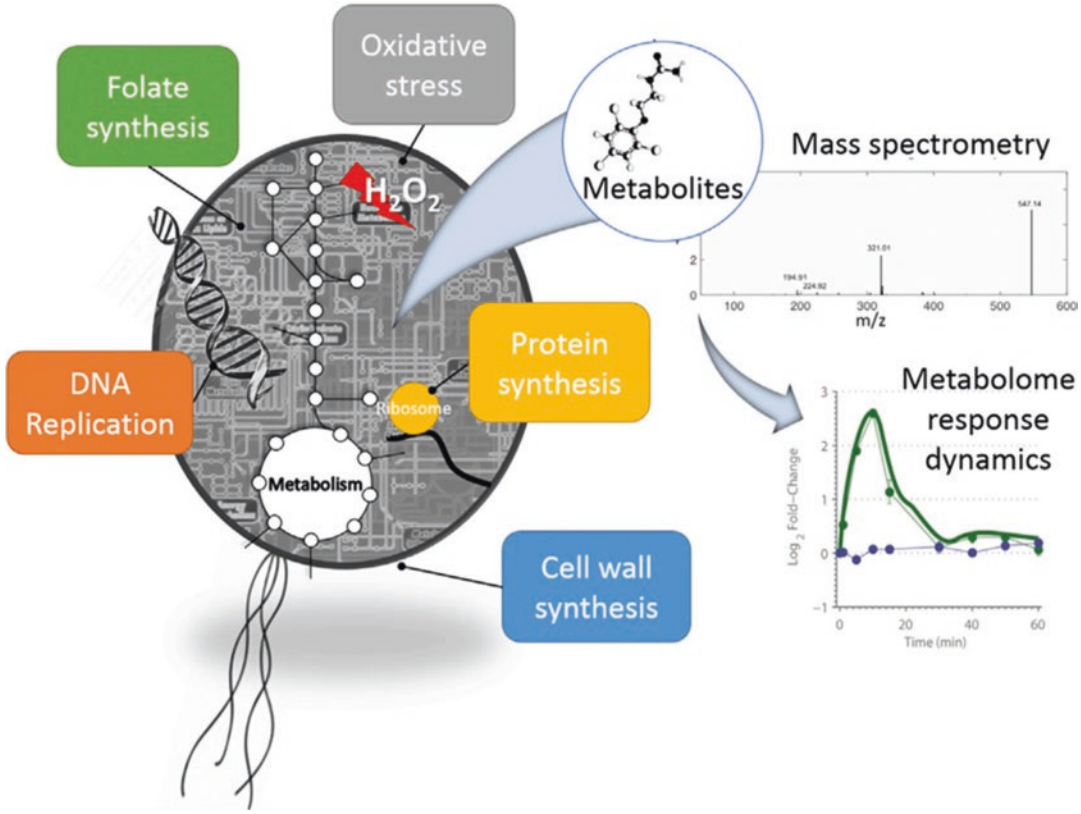

Fig. 7 Monitoring of short-term metabolic changes in *E. coli* after exposure to antibiotics by QTOF-MS

icity and the function of dTDP-rhamnose synthesis in response to quinolone antibiotics [51]. *Klebsiella pneumoniae*, a major pathogen of bloodstream infections, is also an AR-associated strain. Rees and colleagues applied GC × GC-TOFMS to profiling the volatile compounds produced by *K. pneumoniae* in human blood and identified 33 volatile metabolites that are abundant in the pathogenic strain [52]. In addition, *Campylobacter jejuni* (*C. jejuni*), a foodborne microbe, is a great burden on human health due to resistance to antibiotics. UPLC–TOF/MS was used to profile metabolites and discover metabolic signatures associated with chloramphenicol and florfenicol resistance-causing mutations in *C. jejuni*. Up to 41 differential metabolites involved in glycerophospholipid metabolism, sphingolipid metabolism, and fatty acid metabolism were observed in a chloramphenicol-resistant mutant strain of *C. jejuni*. A panel of 40 features was identified in florfenicol-resistant mutants, demonstrating changes in glycerophospholipid metabolism, sphingolipid metabolism, and tryptophan metabolism. This study shows that the UPLC-MS-based metabolomics is a promising and valuable tool to generate new insights into the drug-resistant mechanism of *C. jejuni* [53].

3.4 Treatment of Infectious Diseases Caused by Pathogenic Microbes

Although traditional antibiotics have saved millions of lives and revolutionized the treatment of infectious diseases, side effects associated with the broad use of antibiotics, such as increased

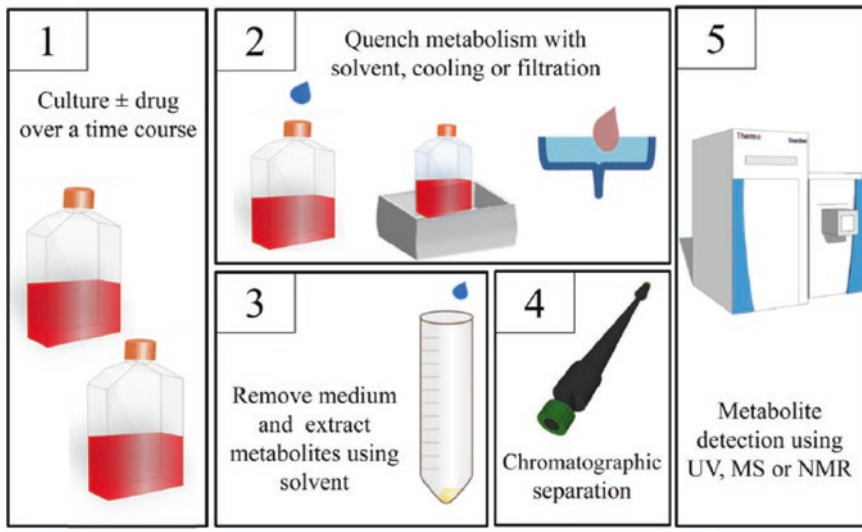

Fig. 8 Microbial metabolomics is applied to investigating the systems actions of antimicrobial drugs involving therapeutic efficiency and toxicology. Microorganisms are grown in the medium with and without drug. Medium is removed; metabolites are extracted from microorganisms and detected by metabolomics methods based on LC-MS, GC-MS, or NMR spectroscopy

emergence of AR and indiscriminate disruption of the beneficial microbiota, have stimulated the need for alternative treatment strategies [54, 55]. One approach is the development of a new generation of antimicrobials that mitigate the spread of AR. Microbial metabolomics could provide the opportunity to understand the biochemistry and pathogenesis of microbial pathogens and facilitate the discovery and development of novel anti-infective drugs [56]. Therefore, investigation of the targets and action modes of drugs via metabolomics can be used to predict the safety and efficacy of a drug (Fig. 8) [57]. For example, a quantitative metabolomics analysis of non-mevalonate isoprenoid synthesis in *Plasmodium falciparum* identified the primary antiparasitic activity of fosmidomycin, and this study will guide future research on the chemical modification of fosmidomycin for treating infections [58]. To investigate the activity of benznidazole, a drug proven to be effective against Chagas disease caused by *Trypanosoma cruzi* (*T. cruzi*), an untargeted LC-MS-based metabolomics approach was developed, and the results revealed that covalent binding of benznidazole with thiols is a primary cause of the drug's activity, which helped us understand the natural variation in *T. cruzi* [59]. A multiomics analysis has facilitated our understanding of the molecular mechanism of eflornithine resistance in African trypanosomes. Metabolic profiling of wild-type *Trypanosoma brucei* (*T. brucei*) and eflornithine-resistant *T. brucei* showed that eflornithine levels were greatly reduced in resistant cells compared to the wild type, and genetic analysis confirmed the role of TbAAT6 (*T. brucei* eflornithine transporter AAT6) in eflornithine action [60]. In addition, triphenylbismuthdichloride (TPBC) has been proven to have toxic effects on many antibiotic-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (*S. aureus*) and vancomycin-resistant enterococci. The use of exometabolomic approaches to monitor metabolic changes in *S. aureus* treated with TPBC showed that this compound has potent antimicrobial activity against many bacterial pathogens, acting by blocking bacterial pyruvate catabolism. Enzymatic studies indicated that TPBC is a highly efficient inhibitor of the bacterial pyruvate dehydrogenase complex [61].

In addition to the development of new antimicrobials, there are alternative approaches for the treatment of infectious diseases, such as photodynamic therapy (PDT), radioimmunotherapy, and bacteriophage treatment. PDT is a technique that combines a nontoxic dye, photosensitizer (PS), and low-intensity visible light in the presence of oxygen to produce cytotoxic species for killing cells [62]. PDT treatment using Green 2 W as the PS has been reported to have a significant effect against *Aspergillus fumigatus* in vitro [63]. Radioimmunotherapy is theoretically useful as an anti-infective therapy against any microbe (including bacteria, fungi, viruses, and parasites) susceptible to radiation. Studies have shown the applicability of radioimmunotherapy to treat *Streptococcus pneumoniae* infections [64]. The efficacy of phages in the treatment of bacterial disease in animal models has been demonstrated, and bacteriophage treatment is a feasible alternative treatment modality for microbe-infected diseases [65]. Nonetheless,

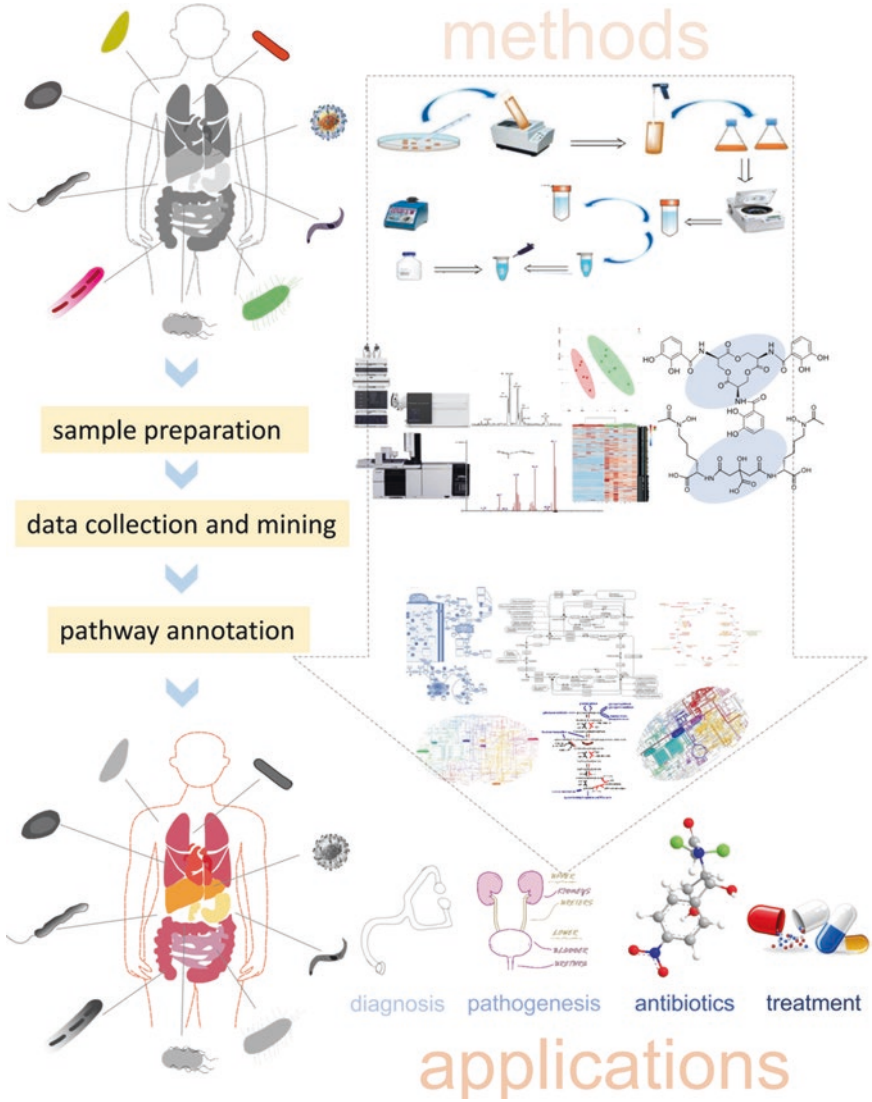

Fig. 9 Overview of microbial metabolomics: from methods to applications

although many therapeutic strategies against infectious diseases have been reported, these strategies remain in the stage of in vitro or animal model experiments.

4 Concluding Remarks and Future Perspective

Microbes contribute to serious infections, such as UTI, CF-associated lung disease, diabetes, and many other diseases, which remain a leading cause of death worldwide. Conventional methods to study infectious diseases, including genomics, transcriptomics, and even proteomics, have presented some shortcomings recently because minor changes in microbiological niches cannot be precisely and directly monitored by these methods. Targeting microbial metabolism has been considered as a promising strategy to solve these problems, because these small changes at gene/protein levels are amplified at metabolite level, which offers valuable information about the functional role of these small molecules in microbial systems. Metabolomics has been widely used to analyze metabolites in biological samples from many sources, including microorganisms [66]. With the advances of MS- and NMR-based metabolomics platforms, microbial metabolomics has been demonstrated as a powerful tool to study microbe-associated infections, particularly the diagnostic biomarkers, pathogenic mechanisms/pathways, antibiotic resistance, and new antimicrobial treatment (Fig. 9).

However, current microbial metabolomics approach has certain limitations. First, the existing methods of metabolite extraction cannot extract all the metabolites of interest from samples. Second, no single analytical instrument alone can perform whole-metabolome profiling. Third, there is no database that contains comprehensive information for all bioactive compounds. Last, but not least, there are challenges associated with the identification of metabolites. To overcome these challenges, combination of microbial metabolomics with other omics technologies, such as genomics, transcriptomics, and proteomics, may become a leading methodology in microbial research.

We hope that this critical review will inspire scientific communities to pay more attention to microbial metabolism from a metabolomics perspective and will significantly advance the discovery and translational applications of microbial metabolomics in clinical diagnosis and pathogenesis, as well as in the discovery of novel therapeutics against a variety of complex infections caused by rapidly expanding microbes.

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