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Review

Recent developments and applications of metabolomics in microbiological investigations

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ABSTRACT

Metabolomics is the comprehensive (qualitative and quantitative) analysis of all metabolites within an organism or a biological system. By studying endogenous metabolites produced from an organism in or around growing biosystems or cells at a given time during growth or the production cycle, metabolomics can potentially provide critical information to help understand the changes occurring in the relevant metabolic pathways. The emerging field of microbial metabolomics has received much attention in recent years, because it not only offers a broad picture of the altered pathways, but also elaborates the mechanisms of the interplay between microbe and host. This article reviews major issues in microbial metabolomics, and gives a comprehensive, critical overview of the current state of the art, future challenges and trends in microbal metabolomics, including systems microbiology and foodomics.

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Abbreviations: CE, Capillary electrophoresis; ECVA, Extended canonical variates analysis; FT-ICR, Fourier transform-ion cyclotron resonance; GC, Gas chromatography; HCA, Hierarchical cluster analysis; LC, Liquid chromatography; LDA, Linear discriminant analysis; MS, Mass spectrometry; NMR, Nuclear magnetic resonance spectroscopy; PCA, Principal-components analysis; PCDA, Principal-component discriminant analysis; PLS-DA, Partial least squares discriminant analysis; QDA, Quadratic discriminant analysis; QIT, Quadruple ion trap; Q-TOF, Quadrupole time of flight; STOCSY, Statistical correlation spectroscopy.

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1. Introduction

To understand the biological mechanism on all different hierarchical levels, systems biology was introduced to examine the structure and the dynamics of cellular and organismal functions, rather than the characteristics of isolated parts of a cell or an organism [1]. Systems biology has now evolved as a broad research field that offers the prospect of assisting in solving fundamental problems [2]. The metabolome is one of the components in systems biology. It is the core in connection with many cellular changes and phenotypes [3]. Specifically, metabolism is regulated by gene expression and post-transcriptional and post-translational events, metabolites are functional entitles within cells and their amounts vary as a consequence of genetic or physiological changes, and can reflect the phenotype (Fig. 1A). Moreover, the metabolome, which refers to the global collection of small-molecule metabolites (such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites) found within a biological sample, is an amplified product of upstream molecular changes in the transcriptome and the proteome [4].

Progress in studies of the metabolome or metabolites has been aided by the recent advent of analytical technologies for comprehensive metabolic analysis, termed "metabolomics" [5]. Historically, there were two terms, "metabolomics" and "metabonomics", which were defined as the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification [6]. The difference between the two terms is simply because of usages amongst different groups that have popularized them. In practice, the term "metabolomics" is often used interchangeably with "metabonomics" [7] (Fig. 1B). Metabolomics is the endpoint of the omics cascade, so it provides access to the biochemical phenotype. Integrative use of multiple approaches in systems biology may thus be a better strategy and can also further offer insight for better understanding of an organism [8].

Metabolomics has proved to be an acceptable, reproducible platform technology, capable of capturing key molecular signatures and characteristics of diseases at different stages and progression [9,10]. Considering the importance of microbes as model organisms, the application of metabolomics techniques in microbiology has recently received much attention. The first microbial metabolomics study was in 1992 by Elmroth et al. [11], who employed GC-MS combined with chemometrics to detect fatty acids, amino acids and sugars in evaluating bacterial contamination of cultures of *Leuconostoc mesenteroides*.

So far, microbial metabolomics has been widely applied in various microbiological fields, such as microorganism identification, mutant screening and functional gene research, metabolic pathway identification and microbial engineering. Compared to studying plants and animals, the main disadvantage of microbe-based metabolomics is that the microbial metabolites are generally complicated and thus difficult to identify. Moreover, the intracellular and extracellular metabolites in microbes are not easily separable. Within a system-wide framework, there are also distinct advantages in studying microbial systems over higher organisms. As microbes are less complex biological organisms, the majority of genome-sequenced data of microorganisms are readily available. Relevant information on gene regulation, metabolic network, and physiology of microbial cells is also easily accessible. Microbiology is therefore a research area that can greatly benefit from recent advances in metabolomics [12].

In the past few years, there have been several excellent reviews on microbial metabolomics [13–18]. However, there is growing awareness that many technical problems in microbial metabolomics have remained unsolved, especially with regards to sample preparation, biomarker identification and mechanism interpretation. There are no reviews providing a comprehensive, in-depth summary of current strategies of microbial metabolomics.

In this context, we seek to focus this review on research methodology and to provide an update on recent progresses and pitfalls of microbial metabolomics. In addition, we also discuss challenges associated with microbial metabolomics.

2. Analytical technologies

Numerous analytical procedures (e.g., sample preparation, signal acquisition, data processing, and data analysis) are involved in a metabolomics study and can directly influence the final results and the biological interpretations. Currently, there are no definitive or standardized operating procedures for microbial metabolomics. Analysis of data from identical samples using different analytical technologies will differentially affect the data and can potentially lead to incorrect findings and contradictory conclusions (Fig. 2A). Numerous analytical technologies were recently developed in several laboratories that promoted the formulation of in-house strategies for sample preparation, and analytical and bioinformatics tools for analysis of the vast amount of data generated [19,20]. Herein, we provide a step-wise description of general analytical technologies for microbial metabolomics.

2.1. Sample preparation

In order to obtain meaningful metabolomics data, microbial metabolomics requires appropriate sample-preparation steps, which include immediate quenching of enzymatic activities, separation of exo- and endo-metabolomes, and the thorough extraction of metabolites.

As intracellular metabolites in microbes may also undergo rapid metabolic responses during the sample-preparation steps (usually in 1–2 s), it is crucial to collect the samples quickly. Such a strategy prevents alterations in the microbial substrate concentrations and helps maintain the stability of the microbial metabolism. Specifically, this step is regarded as especially important for continuous cellular cultivation, as the medium-substrate concentration (such as, glucose) is generally low, so the physiological changes tend to be rapid. To date, some simple instrumentation for collecting samples has been improvised for microbial metabolomics. For example, Schaefer et al. [21] developed an automated sampling device, coupled to a stirred tank reactor, for monitoring intracellular metabolite dynamics with a sampling rate of 4.5 s^{-1}. For determination of rapid changes, Buziol et al. [22] developed a sampling technique based on a continuous stream of biosuspension, from the bioreactor, which was mixed with a glucose solution in a turbulent mixing chamber. In the same year, Visser et al. [23] presented a novel device, namely BioScope, that allowed elucidation of *in vivo* kinetics of microbial metabolism via perturbation experiments.

Generally, the separation of extracellular metabolites and cells should be kept at low temperatures to prevent side reactions. However, the intracellular metabolism is also altered after sampling, due to separation from the culture environment. As current metabolomics focuses on the metabolite *per se*, sample quenching is required to stop the metabolism at specific time intervals to obtain the real metabolic information at given times. The ideal quenching technology should therefore meet two basic criteria:

(1) rapid quenching of enzyme activity;
(2) maintaining the cell or organism intact at all times.

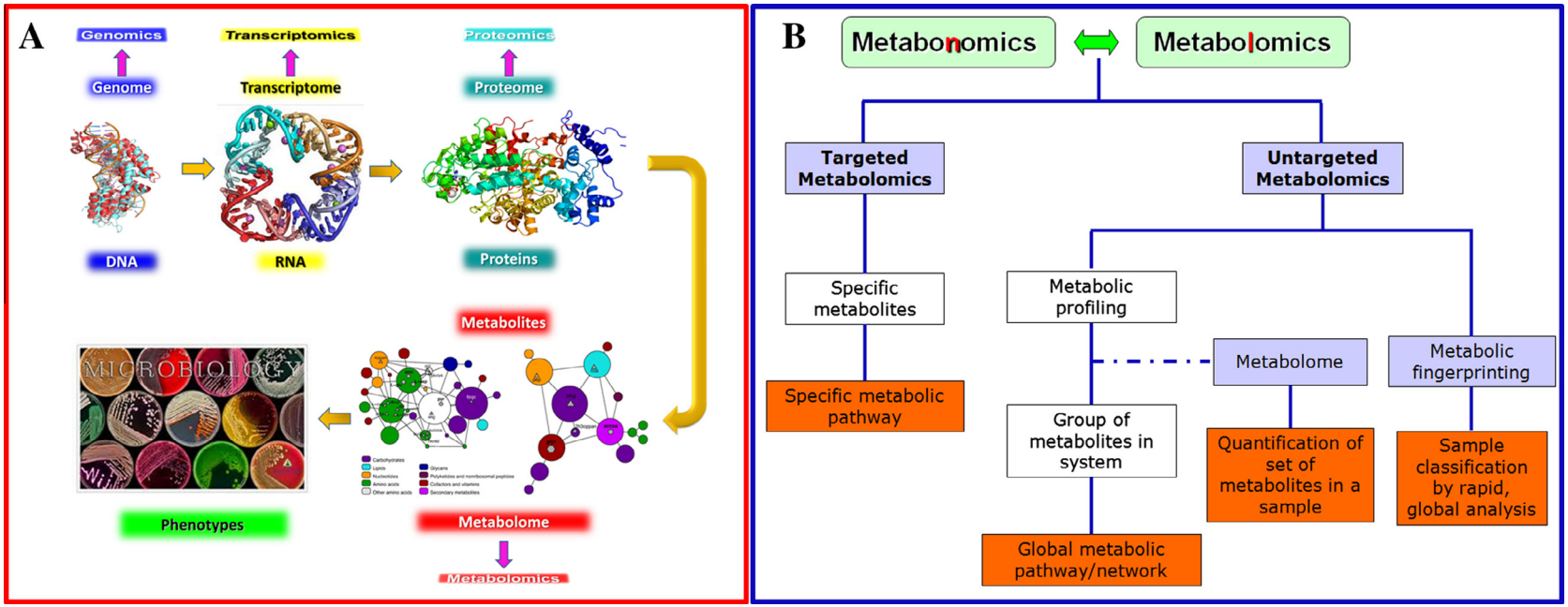

Fig. 1. (A) In information flow in the omics family, the flow is from genes to transcripts to proteins to metabolites and to phenotype. (B) In classification of metabolomics, untargeted metabolomics are generally for discovery of metabolites or global metabolic pathways, whereas targeted metabolomics are tailored for specific metabolic pathway or metabolites.

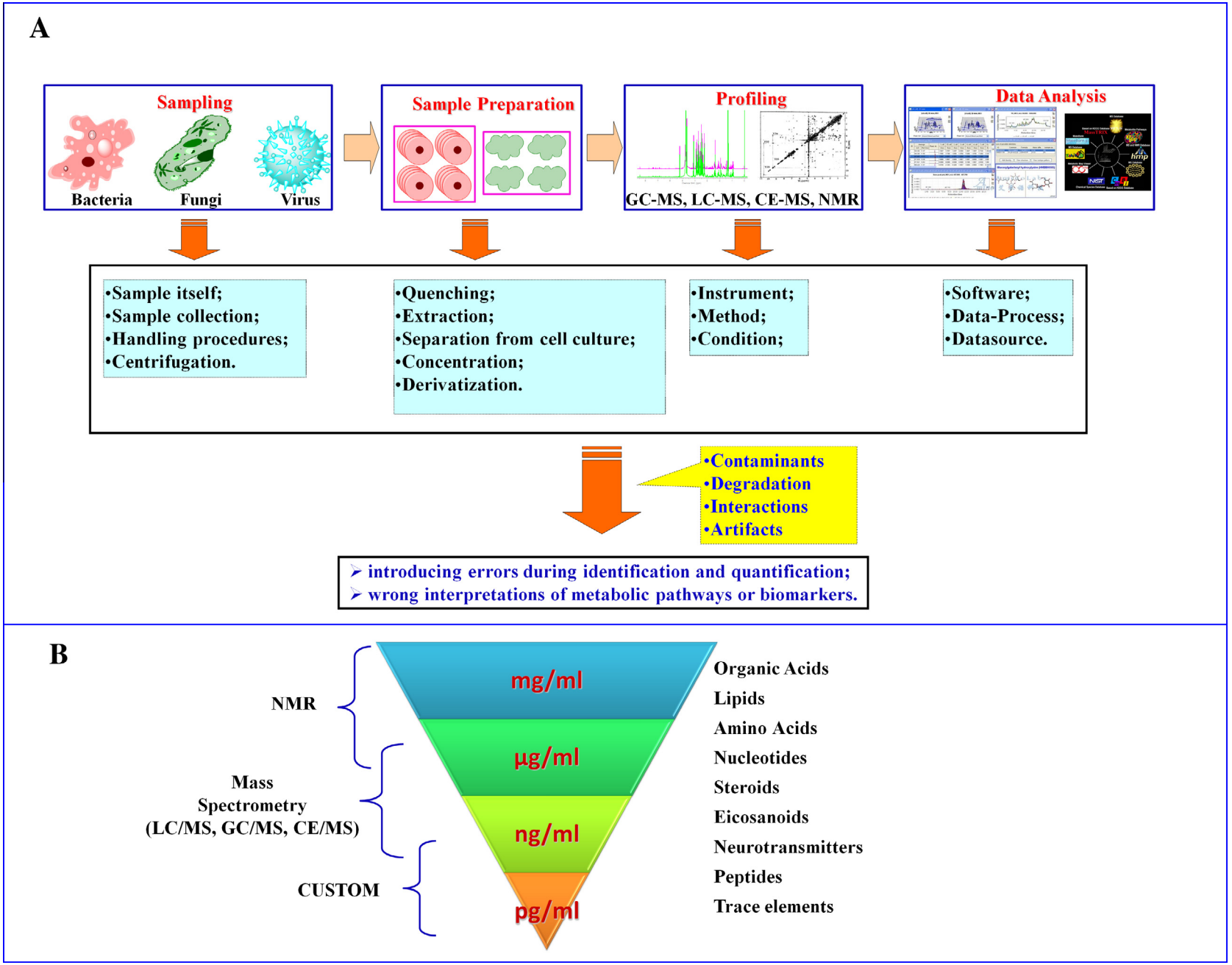

Fig. 2. (A) General strategies and major experimental factors that might lead to variations in microbial metabolomics studies. (B) Current analytical methods that can be used for microbial metabolomics. In general, nuclear magnetic resonance spectrometry covers comprehensive features of the metabolome, whereas mass spectrometry caters for higher sensitivity measurements.

In 1992, De Koning and Van Dam [24] designed the following protocol for quenching of yeast metabolism:

- spraying 15 mL of culture broth into 60 mL of cold (−40°C) 60% methanol in water;
- after cooling for 5 min, the sample/methanol mixture was centrifuged at 5000 rmp for 5 min at −20°C to obtain a cell pellet;
- the cell pellet was subsequently extracted.

This protocol has become one of the most popular quenching methods applied in microbial metabolomics. However, metabolite leakage during quenching accomplished by a fast decrease of the temperature has been attributed to the so-called cold-shock phenomenon, whereby the decrease in temperature results in an increase in the permeability of the cell membrane [25,26].

In order to minimize metabolite leakage, methods and procedures have been developed to quench various specific microbial species. A recent study showed that significant amounts of metabolites can diffuse out of yeast cells into cold methanol. Thus, Spura et al. [27] developed an alternative quenching solution containing 40% ethanol and 0.8% (w/v) sodium chloride at −20°C, proposed specifically for the metabolomics sampling of yeast, and increased detected metabolite levels for 60–80% of 120–160 identified compounds. However, Kim et al. [28] evaluated the quenching method, using 60% (v/v) methanol, pure methanol at −40°C, boiling ethanol (75%, v/v) and fast filtration, by analyzing 110 identified intracellular metabolites of *Saccharomyces cerevisiae*. According to their results, fast filtration, followed by washing with an appropriate volume of water, can substantially minimize metabolite loss.

There are also many quenching procedures applicable to other eukaryotes. Faijes et al. [29] compared four different aqueous quenching solutions for studying *Lactobacillus plantarum*. Only 60% (v/v) methanol buffer containing either 70 mM HEPES or 0.85% (w/v) ammonium carbonate (pH 5.5) in −40°C caused less than 10% cellular metabolite leakage. Compared with the eukaryotic microorganisms, such as filamentous fungi and yeast, bacterial cells are more likely to leak intracellular metabolites, when using cold-methanol quenching. The occurrence of metabolite leakage in Gram-negative bacterium is more frequent than in Gram-positive bacterium, due to compositional differences in their cell-wall structures [30,31]. As a result, a few modified procedures for bacterial quenching have also been established.

In 2008, Winder et al. [32] developed and validated standard operating procedures for quenching metabolites from *Escherichia coli* to determine the best method for global analysis of the *E. coli* metabolome. They claimed that 60% cold-methanol solution (−48°C) is the most appropriate quenching method. However, Taymaz-Nikerel et al. [33] compared a different method of obtaining metabolomics data for *E. coli,* which concluded that cold aqueous methanol was not appropriate, as they observed the release of a major portion of the metabolites from the cells. Hence, Link et al. proposed that the cold mixture of methanol and glycerol (3:2 v/v) at −50°C should be the preferred quenching method for *E. coli* [34].

Moreover, Wu et al. evaluated three quenching methods for *Staphylococcus aureus*, which include quenching-centrifugation (Q-C), filtration-quenching (F-Q) and filtration-quenching-lyophilization (F-Q-L). The study noted that Q-C caused severe metabolite leakage and possible decomposition of nucleotides. F-Q achieved high yields and reproducibility, although it possessed other disadvantages, such as long filtration and rinse times prior to quenching. F-Q-L resulted in losses of several metabolites and a lower yield due to the lyophilization step. Hence, they recommended that F-Q is the most appropriate sampling method because of its high yield and reproducibility [35].

In summary, although numerous quenching methods have been proposed specifically for various microbial species, there is still an unmet demand for a suitable quenching method that can eliminate metabolite leakage, so further efforts are needed to resolve this issue.

Extraction of metabolites is another important process in microbial metabolomics. For the profiling of metabolites, the ideal extraction method should satisfy the following features:

(1) unbiased to include the diversity of metabolites;
(2) maximal extraction of metabolites;
(3) minimal degradation or modifications of physical and chemical properties of metabolites.

Currently, the common extraction methods include the use of cold or hot methanol, hot ethanol, perchloric acid or alkali, a mixture of chloroform and methanol, and acetonitrile. Amongst them, acid or alkali extraction is considered one of the most conventional methods, specifically used for acid- or alkali-resistant metabolites. Perchloric acid has also been widely used to extract bacterial metabolites. This method extracts nucleotides and water-soluble substances with excellent results, such as CAMP, ppGpp and precursors of the cell wall. Similarly, potassium hydroxide was used for extraction of ADP, ATP and precursors. However, low/high pH can cause instability of some metabolites [36]. The methanol-chloroform method is suitable for the extraction of non-polar metabolites, but performs poorly for polar metabolites. Furthermore, the method is relatively time-consuming and the toxicity of the solvents involved is of concern [37]. The methanol- or ethanol-extraction method has the trait of being simple and fast. Furthermore, the extraction agent can be easily removed and metabolites can be concentrated without much difficulty. Nevertheless, hot methanol or ethanol extraction tends to affect the thermal stability of metabolites [38]. However, all existing extraction methods are imperfect for microbial metabolomics, due to their inability to extract all metabolites.

So far, there is a lack of a comprehensive, generally acceptable sample-preparation method that covers all aspects of microbial metabolomics. Due to the diversity of the metabolites, it is difficult to acquire all intracellular metabolites comprehensively by a single extraction method. Thus, the combination of different methods may be useful to improve extraction capability. In short, additional effort to develop an ideal quenching method is needed to improve sample preparation in microbial metabolomics.

2.2. Analytical methods

Global profiling of metabolites requires analytical techniques capable of measuring a variety of chemical classes of molecules in biological samples, potentially existing in a wide dynamic range of concentrations [39]. Two major analytical technologies commonly employed in metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. A number of good review articles were published recently, providing details on technical aspects of MS and NMR, comparing their advantages and limitations, and discussing some of their unique applications [40–43] (Fig. 2B). Herein, we briefly introduce these analytical technologies in microbial metabolomics.

2.2.1. Mass spectrometry for metabolomics

MS is a well-established analytical technique for metabolomics, with high selectivity and sensitivity. Combination with advanced, high-throughput separation techniques, such as gas chromatography-MS (GC-MS), liquid chromatography-MS (LC-MS) and more recently, capillary electrophoresis-MS (CE-MS), have widened the applications of MS technology.

In GC-MS, several hundred compounds could be analyzed simultaneously, including organic acids, most amino acids, sugars, sugar alcohols, aromatic amines and fatty acids. In microbial metabolomics, GC-MS has developed into a mature platform, the first technology used in this field. The Nielsen group has conducted many microbial metabolomics studies based on GC-MS, including:

- the development of chloroformate derivatives for analyzing amino acids for different species of filamentous fungi [44];
- a microwave-assisted derivatization method for analyzing fatty acids in yeast samples [45];
- comparing direct infusion-MS and GC-MS for metabolic footprinting of yeast mutants [46].

However, the derivatization reaction is a prerequisite in GC-MS analysis. The experiment-derived variations and originations of chromatographic peaks are two fundamental issues that could directly influence GC-MS analysis [47,48], so efficient, reproducible derivatization methods are critical elements for GC-MS metabolomics analysis, and can help minimize experimental variations. So far, many strategies have been developed to overcome some of these confounding factors [49–52].

In metabolomics, LC-MS is better suited to the analysis of labile and non-volatile polar and non-polar compounds, without the need for a derivatization reaction. Lv et al. [53] applied an untargeted metabolomics approach to identify compounds, associated with a population with *E. coli*-infected urinary tracts and revealed the discovery of infection-associated endocrine, catabolic and lipid pathways.

Coulier et al. [54] developed an ion-pair LC coupled to electrospray-ionization MS (IP-LC-ESI-MS) method for the simultaneous quantitative analysis of several key classes of polar metabolites. The method was demonstrated to be a useful tool for microbial metabolomics (i.e., a comprehensive platform that could be used to quantitative analysis of a wide range of metabolites in extracts of microorganisms).

Despite being successfully applied in many studies, LC-MS-based microbial metabolomics still has some unsolved problems. High salt concentration produced from culture medium can suppress ESI efficiency and block the smooth flow of the pump, which leads to poor quantitation and compromises the repeatability of the LC-MS analysis.

Recently, a new method was developed for the quantitative analysis of the microbial metabolome using a mixture of fully-uniformly (U) (13)C-labeled metabolites as internal standards in LC-MS analysis. This method has led to reduced workload and increased robustness, and made metabolomics analysis more precise [55].

CE-MS separations are very efficient, require only small amounts of sample, regents and solvent, and are relatively inexpensive (cheap fused-silica capillaries *versus* costly LC columns). However, reproducibility tends to be one of the major challenges, due to the small size of the samples and analytical conditions. Soga et al. [56,57] developed and utilized CE-MS to determine intracellular metabolites extracted from wild-type and gene-knockout mutants of *E. coli* and *Bacillus subtilis*. The applicability of CE-MS for microbial metabolomics studies has been summarized in several reviews [58–60].

2.2.2. Nuclear magnetic resonance spectroscopy for metabolomics

NMR spectroscopy is one of the principal techniques used to identify organic compounds distinctly. NMR-based metabolomics can provide a 'holistic view' of metabolites under certain conditions, due to its ability to measure intact biomaterials non-destructively, while obtaining rich structural information. Disadvantages of NMR technology include low sensitivity and spectral resolution, and poor time resolution. It also suffers from limited quantification methods and limited availability of commercial software [61].

In microbiology, the composition of intracellular metabolites of microbe is complex and includes inorganic ions, hydrophobic lipids and complex natural molecules. Their molecular concentrations may range and alter over several orders of magnitude (from pmol to mmol). For this reason, concerns over the low sensitivity of NMR have greatly limited its widespread use in microbial metabolomics. However, there is still much interest in applying NMR techniques for microbial metabolomics. For example, Boroujerdi et al. [62] have examined intracellular metabolites from bacterium *Vibrio coralliilyticus* cultured at 27°C (virulent form) and 24°C (avirulent form) using NMR technology.

2.3. Data processing and analysis

Data preprocessing is an intermediate step between raw spectra and data analysis. Typical data processing usually proceeds through multiple stages, including baseline correction, feature detection, filtering, alignment and normalization. So far, software has been developed to convert the initial MS raw data to a two-dimensional data table {e.g., MZmine [63], XCMS [64] and MET-IDEA [65]}. Many instrument manufacturers also offer their own proprietary software {e.g., MarkerLynx (Waters), MassProfiler (Agilent), MarkerView (Applied Biosystems/MDS SCIEX) and SIEVE (Thermo Fisher Scientific)}. For NMR-data preprocessing, quantitative or scored integrals of specific spectral peaks called "binning" are used [41].

Multivariate and univariate statistical methods are professional approaches to analyzing and maximizing information retrieval from complex raw data. They can be classified into two sub-families of methods, namely unsupervised and supervised. There are several unsupervised approaches available, including principal-components analysis (PCA), robust-PCA, hierarchical cluster analysis (HCA), K-means, statistical correlation spectroscopy (STOCSY) [66,67], and cluster analysis statistical spectroscopy (CLASSY) [68]. Supervised methods can reveal the most important factors of variability characterizing the data set, which are commonly found to be distinct from the factor specifically investigated. Many other discriminant techniques are available to analyze metabolomics datasets, including partial least squares discriminant analysis (PLS-DA) [69], orthogonal PLS-DA (OPLS-DA) [70], linear discriminant analysis (LDA), quadratic discriminant analysis (QDA) [71], principal-component Discriminant Analysis (PCDA) [72], and extended canonical variates analysis (ECVA) [73].

Biomarker identification is another essential component of metabolomics analysis and is a challenging, time-consuming process, especially in microbial metabolomics. This is because the microorganism can produce a very diverse range of metabolites under different conditions, which may not be commonly recognized by standard spectrometry. However, the "naïve" identification of metabolites by simplistic mass matching, without considering their molecular origins, can lead to spurious, humorous or meaningless compound identifications [74]. Benefitting from the wealth of information, a number of microbial databases are emerging and have helped to speed up identification. In parallel, researchers can also resort to general metabolomics databases. The representative useful databases are shown in Table 1.

In microbiology, metabolic pathway analysis may shed light on the interaction of metabolites and complement functional genomics research exploring in gene-expression data. However, the generalized metabolic pathway, such as KEGG, is unable to cover comprehensively the entire metabolic network and the intricate metabolic processes of all metabolites. In view of this, silico models of microbial network have been constructed. Oh et al. [75] presented a genome-scale reconstruction of *B. subtilis* metabolism

Table 1
Representative website resources for microbial metabolomics

Microbial metabolomics resources
HMP: The Human Microbiome Project http://www.hmpdacc.org/ EcoCyc: Encyclopedia of *Escherichia coli* K-12 Genes and Metabolism http://www.ecocyc.org/ ECMDB: The *Escherichia coli* Metabolome Database http://www.ecmdb.ca YMDB: The Yeast Metabolome Database http://www.ymdb.ca NMD: National Microbiological Database http://www.foodsafety.govt.nz/industry/general/nmd/ MNPD: Microbial Natural Products Database http://naturalprod.ucsd.edu/ UMBBD: University of Minnesota Biocatalysis/Biodegradation Database http://umbbd.ethz.ch/ BioCyc Pathway/Genome Databases and Pathway Tools Software http://biocyc.org/
Metabolomics databases
HMDB: http://www.hmdb.ca/ BiGG: http://bigg.ucsd.edu/ SYSTOMONAS: http://systomonas.tu-bs.de/ LIPID MAPS: www.lipidmaps.org/ KEGG: www.genome.jp/kegg/ PubChem Compound: http://www.ncbi.nlm.nih.gov/pccompound MetaCyc Encyclopedia of Metabolic Pathways: http://metacyc.org/

and its iterative development based on the combination of genomic, biochemical, and physiological information and high-throughput phenotyping experiments, consisting of 1020 metabolic reactions and 988 metabolites. In 2008, Herrgård et al. [76] produced a consensus metabolic network reconstruction for *S. cerevisiae*, including 1168 metabolites, 832 genes, 888 proteins and 1857 reactions.

3. Microbial metabolomics and applications

Microbial metabolomics is one of the platforms for integrating biological information into systems microbiology [77]. Microbial metabolomics has contributed to the discovery of unique metabolic pathways, regulatory interactions and homeostasis mechanisms. Recently, many metabolic pathways were revealed by investigations in microbial metabolomics [78–80]. Meanwhile, metabolomics has also made substantial contributions to mapping novel substrates and metabolic pathways, and is a promising quantitative tool for investigating the dynamics of microbial evolution and development, rather than being used simply for resolving connections between species [81]. These findings can provide useful insights into global metabolism and metabolic regulation. In other words, microbial metabolomics can contribute significantly towards understanding systems biology. To date, there are many topics and applications reported in the literature that have demonstrated such capability. In this review, we further select the following four areas to illustrate recent progress in microbial metabolomics.

3.1. Interactions of gut microbiota

The amount of microbes in humans far exceeds that of their own cells [82]. Amongst these microbes, the gut microbiota is one of the topics of current focus in systems biology and metabolomics. Indeed, the gut-microbiota community is one of the best illustrations for interactions between mammal and microbial metabolomes. However, this does not undermine the biological importance of other microbial communities with respective colonization sites in our body. Promising and interesting findings on other microbiota have also been observed [83–85]. Gut microbiota possess a broad spectrum of biological effects on health, such as preventing the development of serious diseases, improving the immune system and gut health, through to stimulating the brain centers responsible for appetite and food-intake control [86].

Normally, the gut microbiota offer many benefits to the host, including defense against pathogens, immunity, renewal of gut epithelial cells, harvesting inaccessible nutrients, and generating energy by anaerobic metabolism of substrates [87]. The microbes residing in the gut are considered an important integral part of the intestinal ecosystem [88,89]. Hence, the analysis of metabolites derived from gut microbe–host co-metabolism is essential for understanding the metabolic functions of the gut microbiome.

Recent studies revealed that the gut microbiota has many potential biological effects in the host metabolism. Nicholson et al. [90] employed multiple technologies to investigate microbiome-mammalian metabolic interactions in a mouse model, including metabolic profiling by ^{1}H-NMR of liver, plasma, urine and ileal flushes, and targeted profiling of bile acids by LC-MS and fatty acids in cecum by GC equipped with a flame-ionization detector (FID). They showed that the microbiome modulates absorption, storage and harvest of energy from the diet at the systems level. Furthermore, they confirmed that the microbiome strongly impacts upon the metabolism of bile acids, and the gut microbiota can modulate metabotype expression at the levels of both local (gut) and global (biofluids, kidney, liver) systems using NMR technology [91].

Other research groups have also demonstrated that gut microbes can influence many important metabolic processes in the host. For example, Wikoff et al. [92] reported a broad MS-based metabolomics study that demonstrated a surprisingly substantial effect of the gut microbiome on mammalian blood metabolites. Saric et al. [93] presented a ^{1}H-NMR-based metabolomics investigation in aqueous fecal extracts of humans, mice and rats, which revealed differential gastrointestinal microbiome influences on the fecal metabolome. The main chemical classes involved in the microbiome-mammalian metabolism include lipid, choline, bile acids and fatty acids [94,95]. However, it is likely that there are many more classes of compounds and interactions to be discovered, considering the vast diversity of gut microbial genes with unidentified functions.

Generally, modulation of the gut-microbiota composition has potential beneficial effects in reducing occurrence or in control of disease manifestation. However, the gut-microbial community can also exert adverse effects on hosts, resulting in various immune disorders within and outside the gut [96]. Alteration or instability of the microbiota or changes in microbiota biodiversity is a characteristic phenotypic hallmark of a number of gastrointestinal disorders and metabolic diseases. Irritable bowel syndrome (IBS) and Crohn's disease (CD) are perhaps the best known examples of inflammatory intestinal diseases resulting from a dysregulated immune response to the gut microbial community. Ahmed et al. [97] reported a GC-MS based metabolomics study of the fecal volatile organic metabolites (VOMs) in patients with diarrhea-predominant IBS, active CD, ulcerative colitis and healthy controls. Short-chain fatty acids, cyclohexanecarboxylic acids and aldehydes were observed in the study to be associated with IBS. An NMR-based metabolomics study suggested that, in a subset of IBS patients, there exists potential dysregulation in energy homeostasis (serum glucose) and liver function (serum tyrosine) that may be improved through probiotics supplementation [98].

Some of these recent studies clearly indicated that metabolomics is an emerging novel technology that can provide exclusive insights into the role of gut microbial metabolic interactions in an individual's susceptibility to health and disease outcome. A recent review article highlighted the critical interactions of gut

microbiota with their host, and some examples of metabolomics applications for characterizing the metabolic footprints of the gut microbial-host co-metabolism [99].

3.2. Food, nutrition and microbial metabolomics

Rapidly emerging discipline "foodomics" examines the connections of food components, foods, diet, individual, health, and diseases by applying advanced omics technologies in order to improve individuals' health, well-being, and knowledge [100]. Foodomics has been defined as a discipline that integrates the food domain and the nutrition domain as a whole, using tools from genomics, transcriptomics, epigenomics, proteomics, and metabolomics [101].

As an integral part of the omics family, metabolomics is widely used in foodomics. To date, microbial metabolomics has also been successfully applied to several foodomics or nutritional studies, such as food safety, food quality and food nutrition.

For years, food safety has been a legitimate concern. The advancement of metabolomics technology allows simultaneous and sensitive quantification of diverse groups of compounds in different food matrices. The representative researches on the simultaneous analysis of more than 30 pesticides and/or antimicrobials was summarized in a recent review [102].

Pathogens, toxins, and sub-products in food degraded by microorganisms are relevant aspects of food safety. Hence, investigations on relevant metabolites are also important to food safety. MS-based technologies have made great efforts to detect toxins from food microorganisms {e.g., GC-MS has been used to profile volatile metabolites related to a particular microbial contamination [103–105], and NMR technologies to determine microbial toxins [105–108]}.

Wholesomeness is a characteristic quality of food that is acceptable to consumers, so metabolomics technologies are widely used in food-quality inspection. For example, MS technologies were used for analysis of genetically-modified organism (GMO) crops, providing very useful information on GMO composition (e.g., metabolites and proteins) [109].

NMR was introduced to elucidate the variation between cultivars and the magnitude of changes in the metabolic fingerprints between harvests from spring season and the second growing season [110].

Also, food composition has the potential to improve health or even to prevent disease. Recent nutritional metabolomics studies inspected gut microbiota–host metabolic interactions with a specific focus on the co-metabolism of food composition [111]. Microbial metabolomics has been used to characterize the effects of both deficiency and supplementation of different nutrients, and to compare the metabolic effects of closely-related foods. It may also enable more accurate measurements of diet-linked effects on an individual or a community, while minimizing the effects of confounding factors, such as age, gender, physiological states and lifestyle [112]. For example, Ibáñez et al. [113] developed an analytical multiplatform that combined CE, RP/UPLC and HILIC/UPLC, all coupled to TOF-MS, to study the anti-proliferative effects of dietary polyphenols on human colon-cancer cells. Their results indicated the intracellular levels of 22 highly-related metabolites, known to be essential for the maintenance of the cellular functions, were altered in HT-29 cells by treatment with dietary polyphenols.

An NMR-based metabolomics study exploring urinary profiles from control-fed and diet-restricted dogs found that diet restriction altered the activities of the gut microbiota, manifested by variation in aromatic metabolites and aliphatic amine compounds [114].

Another NMR-based metabolomics study successfully demonstrated that different weaning diets in pigs induced divergent and sustained shifts in the metabolic phenotype, which resulted in the alteration of urinary gut microbial co-metabolites, even after 4 weeks of dietary standardization. Also, the production of gut mucosa-associated IgA and IgM was found to depend upon the weaning diet and on *Bifidobacterium lactis* supplementation [115].

3.3. Phytopathogens and entomopathogens

Although metabolomics studies on plant pathogens have only started emerging, their potential to dissect many facets of the pathogen will quickly promote an increase in popularity. This sub-section describes the applications of metabolomics to study phytopathogens and their respective diseases.

MS is the most commonly-used technique for metabolomics in fungal phytopathogen research. While GC-MS is the predominant technique, LC-MS has not been applied in any comprehensive metabolomics study but has been used to examine and to screen for secondary metabolites [116]. Nevertheless, MS-based metabolomics is a robust, sensitive and reproducible analytical technology to study fungal phytopathogens. For example, GC-MS profiling discovered that mannitol have critical roles during pycnidial development of wheat pathogen *Stagonospora nodorum* [117]. GC-MS profiling was also used to identify significant alterations in metabolism upon exposure of *S. nodorum* to 0.5 M NaCl. It observed that arabitol and glycerol responded to salt stress in pathogens. Moreover, a reverse genetic approach was undertaken to dissect the role of the arabitol metabolism. This study is pertinent example of metabolomics contributing to the direct identification of gene functions and involvement [118].

Metabolomics has also been used to help dissect the mutation of pathogens. Metabolic profiling was undertaken on the mutant strains of *S. nodorum* lacking the *Sch1* gene to help elucidate the roles and the functions of *Sch1* gene. GC-MS analysis could identify secondary metabolites in *Sch1*-absent mutants at 200-fold concentrations greater than wild-type strains. ESI-MS/MS was then utilized to confirm the identity of the altered molecule as alternariol [119].

In addition, metabolomics has been used as a tool for the discrimination of fungal diseases on infected hosts. Several studies have exhibited that certain volatiles can be associated with a single disease. Cao et al. used LC-MS to characterize the metabolites of three different host tissues in infected and uninfected ryegrass, and revealed the presence of several compounds in the infected plants, including the ubiquitous mannitol, as well as peramine and perloline [120].

The application of NMR to dissect fungal plant diseases is in its early stages, with only a handful of reports having emerged so far. Keon et al. [121] used ^{1}H-NMR to study the nutritional content of the apoplast in wheat during infection by *Mycosphaerella graminicola*.

Entomopathogen is another object in microbial metabolomics. Traditionally, most of the investigations on the interactions of entomopathogens and hosts focused on the levels of genome and transcriptome [122–124]. Recently, there were also some reports about metabolomics studies of entomopathogens {e.g., Luo et al. [125], who investigated the global metabolic profiling of mycelia and conidia of transgenic and wild-type *Beauveria bassiana* using LC-MS analysis}.

4. Recent trends

Corresponding to the rapid development of analytical technology, microbial metabolomics has also made significant progress in the past two decades with exciting discoveries relating to altered metabolism and regulation of biological systems, as

detailed above. However, there are certain problems that remain to be resolved.

First, there are no standard protocol in microbial metabolomics for the instantaneous quenching of microbial metabolic activity, comprehensive extracting of metabolites and analysis of the metabolites of interest. Current quenching procedures appear to be strongly dependent on a specific organism, and incapable of preventing metabolite leakage, so, when developing a quenching protocol for precise quantification of intracellular metabolites, the metabolite-leakage test is an essential experimental consideration due to the inherent differences in cell-wall structure and membrane composition in different organisms. Recent extraction methods are most suited to the analysis of certain classes of metabolite and can be further improved. Hence, comprehensive metabolite profiling may require the combination and the integration of multiple extraction methods.

Second, although a number of databases for microbial metabolomics are emerging, they are limited to specific microbes, particularly yeast and *E. coli*. There are also no available databases that encompass integrated metabolic data from different microbes, especially for viruses, bacteria and fungi. This may be due the boundless diversity and complexity of microbial metabolites. Indeed, hundreds of thousands of compounds have been identified from microorganisms, and their numbers are still increasing. As a result, the construction of a comprehensive database is a major venture that would entail enormous efforts, and technological and financial resources.

In addition, it seems that mammalian metabolic pathways cannot be entirely applied to microorganisms, as many microbial reactions do not exist in mammals, such as secondary metabolite biosynthesis. As existing pathway data cannot fully fulfil the needs of microbial metabolomics, it is necessary to identify and to establish specific distinctive metabolic pathways and networks for microorganisms.

Furthermore, recent research has mostly focused on those model microbes. Increasingly, genomic information from non-model microbes has been obtained. However, these microbes are relatively less studied and the genomic information is also less employed to direct metabolomics studies.

Last, current microbial metabolomics studies have largely been concerned about metabolites solely, with little regard for their origins. For example, glucose from microbe and host is chemically identical, but the biological significance and the mechanisms that lead to their differential regulation differ. Hence, it is important to study metabolic changes of microbe and host differently, especially in research on microbe–host interactions.

In conclusion, the current progression of microbial metabolomics is still not flawless and will require further development.

Compared to the application of metabolomics in pharmacological drug research and development, disease diagnosis and plant metabolomics, microbial metabolomics is still in its early stage of development. Nevertheless, microbial metabolomics does possess its own strengths, such as simplicity of the microbial system, an abundance of microbial genomic data and rich knowledge of microbial metabolic network and physiological characteristics. Based on the above merits, microbial metabolomics has been widely used to identify new metabolites, studying the environmental effects and elucidating novel and complex metabolic networks and pathways. We can therefore examine the reaction kinetics, the metabolic pathways and the mechanisms on a specific metabolic reaction, using both qualitative and quantitative analysis of metabolomics. Furthermore, the biomarkers and metabolic pathway derived from metabolomics investigation can also complement other omics tools to understand alterations in the global organism. Notably, microbial metabolomics alone is inadequate to understand cellular metabolic activity. Thus, a systems microbiology approach, integrating genomics, transcriptomics, proteomics and metabolomics, is needed for the discovery of metabolic pathways, regulatory interactions and homeostasis mechanisms. Correspondingly, the other omics techniques can offer many useful molecular leads for understanding metabolomics data, especially with targeted investigation. Moreover, due to the simplicity of the microbial system, genetic and proteomic techniques (e.g., knock-in/out and overexpression) can easily be applied for validation of a metabolomics result.

5. Conclusion

As highlighted above, microbial metabolomics has opened up a new area of scientific interest for microbiological study and is rapidly becoming an emerging research field. It has made tremendous contributions to the development of systems microbiology. Being both an important element of systems microbiology and an invaluable technological platform, microbial metabolomics will inevitably soon become an essential and exceptional field of focus in systems microbiology.

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