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Review on microbial metabolomics of probiotics and pathogens: Methodologies and applications

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Abstract: In recent years, microbial metabolomics, a new field that has attracted wide attention, provides a map of metabolic pathways and clarifies the interaction mechanism between microorganisms and hosts. Many microorganisms are found in the human intestine, oral cavity, vagina, etc. Probiotics could maintain the good health of the host, while pathogens and an imbalance of bacterial flora lead to a series of diseases of the body and mind. Metabolomics is a science for qualitative and quantitative analysis of all metabolites in an organism or biological system, which could provide key information to understand the related metabolic pathways and associated changes. This approach analyzes the final products of cellular regulatory processes, the level of which can be regarded as the ultimate response of the biological system to genetic or environmental changes. Microbial metabolomics has been widely used in different research fields, such as microbial phenotypic classification, mutant screening, metabolic pathways, microbial metabolic engineering, fermentation engineering monitoring and optimization, microbial environmental pollution, and so on. However, there are only a few reviews on microbial metabolomics of probiotics and pathogens. This review summarizes the main methodologies, including sample preparation, identification of metabolites, data processing, and analysis. Recent applications in microbial metabolomics of probiotics and pathogens are also described. This paper first summarized the research progress and application of microbial metabolomics from two aspects: probiotics and pathogenic bacteria. Probiotics and pathogenic bacteria do not exist independently most of the time; hence, these were reviewed in the research field of coexistence of probiotics and pathogenic bacteria, which was subdivided into important microbial research fields closely related to human health, including the human gut, oral cavity, food, and nutrition-related microorganisms. Then, the main problems and trends associated with microbial metabolomics are discussed.

Introduction

Microbial metabolomics, as a new field, could provide a map of metabolic pathways and clarify the mechanism of interaction between microorganisms and hosts (Fobofou and Savidge, 2022; Joshua, 2019). There are many microorganisms in the human intestine, oral cavity, and so on (Moreno *et al.*, 2022; Vlachojannis *et al.*, 2018) (Fig. 1). Whether in quantity or gene, the microbes parasitizing on the surface and inside of the human body are far more than the human body cells (Ke *et al.*, 2018b). Of the trillions of microorganisms, more than 1,000 species of bacteria live in the intestine and possess more than 3 million genes (Sebastian Domingo and Sanchez Sanchez, 2018; Zhu *et al.*, 2015). The intestine carries up to 2 kg of microorganisms (Macfarlane and Macfarlane, 1997; Mondot and Lepage, 2016). Two-thirds of an individual's intestinal flora is unique to oneself; even in identical twins, the composition of bacterial communities is not exactly the same. Probiotics could maintain the health of the host, while pathogens and imbalance of bacterial flora lead to a series of diseases of the body and mind (Lukic *et al.*, 2017; Meng *et al.*, 2018; Gupta *et al.*, 2021; Manole *et al.*, 2021). Bacteria existing in the human body possess a wide range of functions, such as affecting fat storage, angiogenesis, and immune system, regulating the nervous system and bone mineral density, protecting epithelial cells from injury and pathological tolerance, degrading food components, biosynthesis of vitamin and amino acids, and metabolism of drugs and so on (Bliss and Whiteside, 2018; Boursi *et al.*, 2018) (Fig. 2).

Metabolomics is a science for qualitative and quantitative analysis of all metabolites in an organism or biological system, which could provide key information to people to understand

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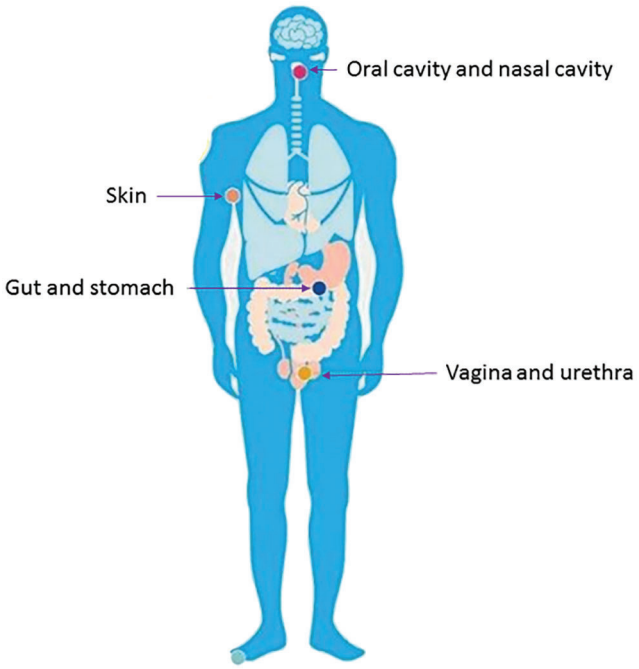

FIGURE 1. Distribution of microorganisms in the human body. A large number of microorganisms are distributed in the human skin, oral cavity, gastrointestinal tract, respiratory tract, genitourinary tract, etc.

the related metabolic pathways and their changes. This approach analyzes the final products of cellular regulatory processes, the level of which can be regarded as the ultimate response of the biological system to genetic or environmental changes. Due to the importance of microorganisms in biological systems, metabolomics technology has attracted wide attention in microbial research (Baidoo and Teixeira Benites, 2019; Joshua, 2019; Mousavi *et al.*, 2019; Murovec *et al.*, 2018). In 1992, Elmroth *et al.* (1992) assessed microbial metabolomics for the first time (Marciniec *et al.*, 1992). Fatty acids, amino acids, and carbohydrates were detected by gas chromatography-mass spectrometry to evaluate bacterial contamination of *Leuconostoc mesenteroides* during their culture.

Initially, microbial metabolomics was mainly used for the biological identification of different strains (Boersma *et al.*, 2001; Koek *et al.*, 2006; Mashego *et al.*, 2007; van der Werf *et al.*, 2005). Over recent years, microbial metabolomics has been widely used in different research fields, such as microbial phenotypic classification, mutant screening, engineering of metabolic pathways and microbial metabolism, monitoring and optimization of fermentation engineering, monitoring microbial environmental pollution, and so on (Covington *et al.*, 2017; Jacobs *et al.*, 2016; Joshua, 2019; Sumner *et al.*, 2015). The precise, sensitive and high-throughput research methods of microbial metabolomics are the basis of microbial metabolomics research (Mousavi *et al.*, 2019). However, the methodology and applications of microbial metabolomics have been rarely reviewed. This review summarizes the main methodologies and applications of probiotics and pathogens in microbial metabolomics, and discusses the main problems and trends of microbial metabolomics.

Methodology of Microbial Metabolomics

In the studies of microbial metabolomics, the main research methods are roughly the same, as described below (Fig. 3).

Sample preparation

To obtain meaningful information, microbial metabolomics research needs to adopt appropriate sample preparation steps, including rapid sampling, quenching, and extraction of the metabolite. Rapid sampling prevents the dramatic

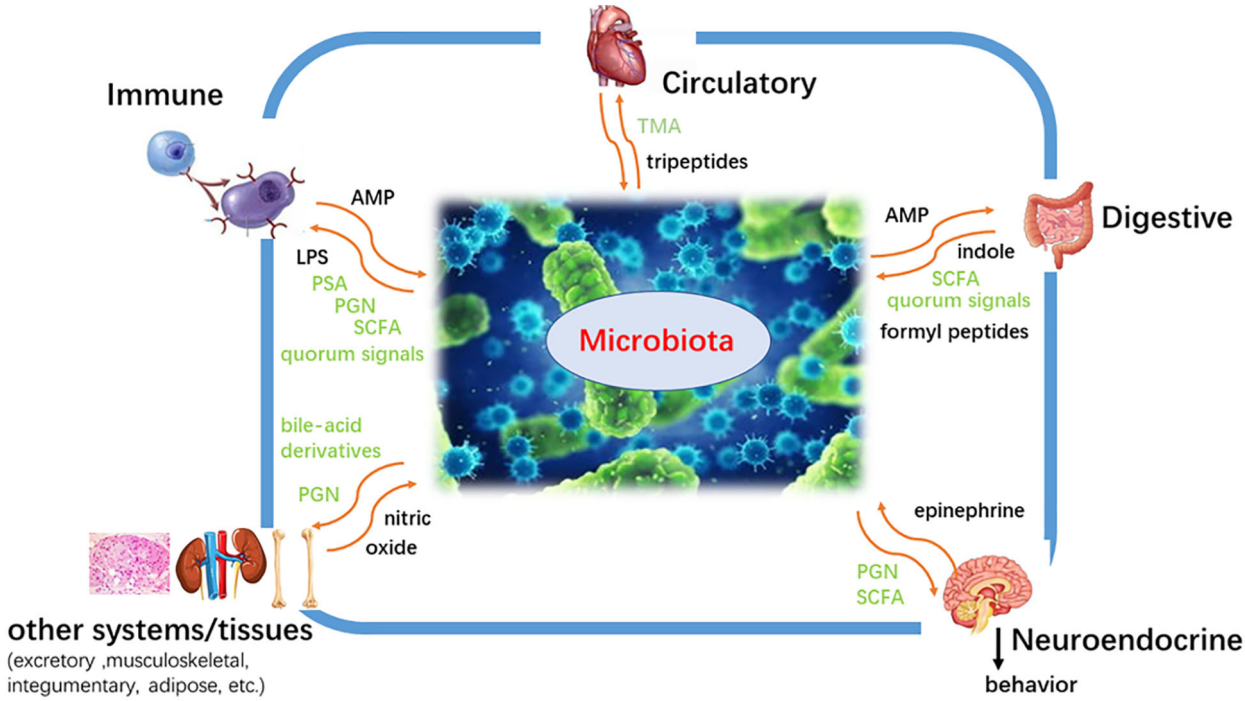

FIGURE 2. The effect of microorganisms on the human body. Microorganisms exert a wide range of functions, including affecting fat storage, angiogenesis, and immune system, regulating the nervous system and bone mineral density, protecting epithelial cells from injury and pathological tolerance, degrading food components, biosynthesis of vitamin and amino acid, metabolic drugs and so on.

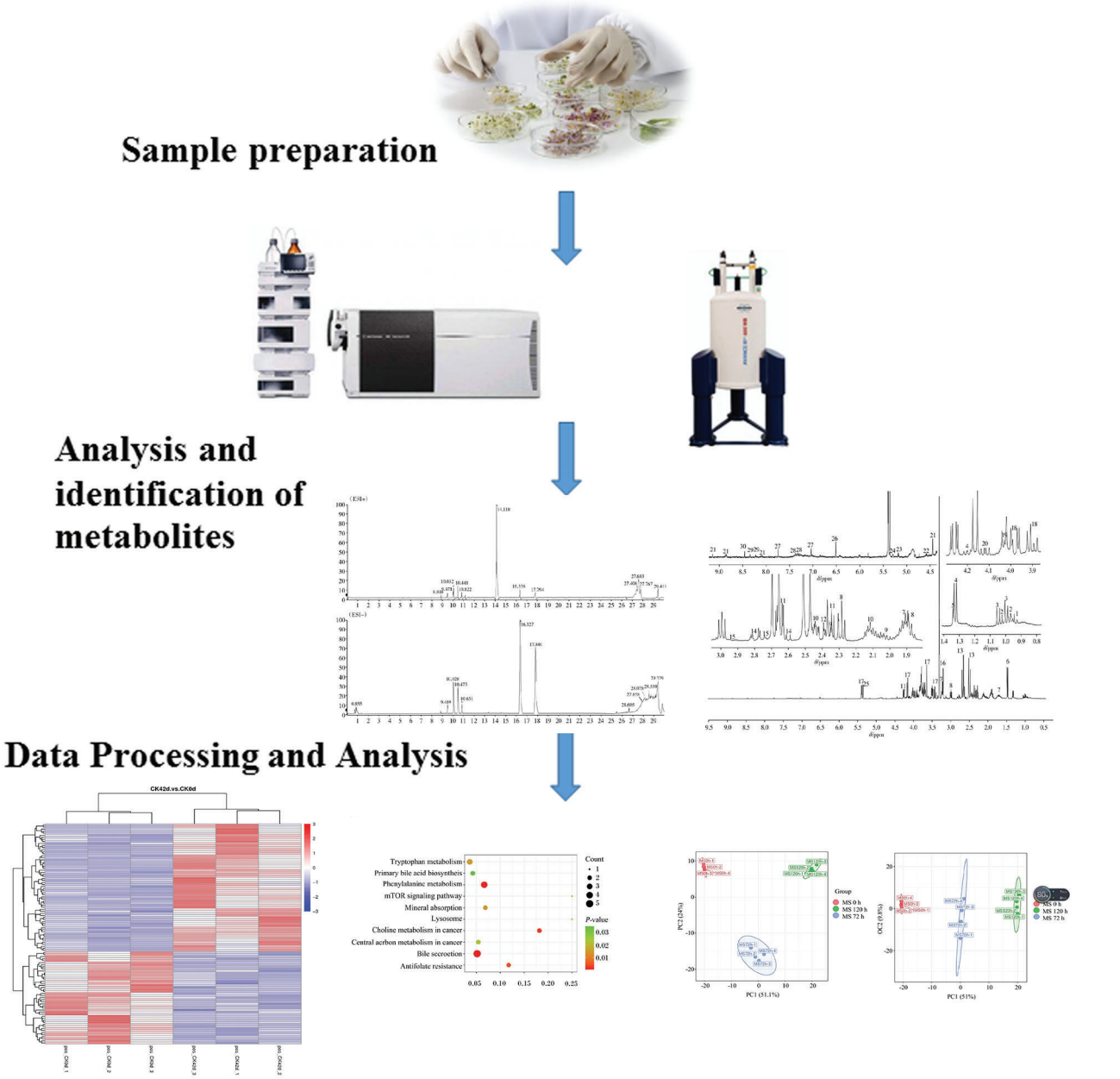

FIGURE 3. A broad schematic for research on microbial metabolomics. The main research workflow involves sample preparation, analysis and identification of metabolites, and data processing and analysis.

change in the concentration of substrate and helps to maintain the stability of microbial metabolites. Therefore, many simple sample collection devices, such as the BioScope device and Fast Swinnex filtration (FSF) device, are often used to achieve rapid sample collection (Zhu *et al.*, 2013).

To ensure the acquisition of the information of the sample at a specific time, it is necessary to quench the sample quickly to stop the metabolic reaction. The ideal quenching technology should quench the enzyme activity quickly and maintain the integrity of the cell or organism (Almanza-Aguilera *et al.*, 2017; Joshua, 2019; Mayta-Apaza *et al.*, 2018; Teoh *et al.*, 2016). However, many quenching methods, such as organic solvent quenching, could destroy cell walls and cell membranes, leading to a significant leakage of intracellular metabolites (Mojica *et al.*, 2008; Nandakumar *et al.*, 2000). For example, methanol or ethanol as a quencher may cause the leakage of some metabolites (Wang *et al.*, 2017a). At present, flow cytometry is used to evaluate the degree of cell membrane damage caused by different quenching methods. The quenching of *Escherichia coli* by normal saline at −80°C results only in 6% damage to the cell membrane, while conventional methanol quenching causes only 1/10 of the cell membrane damage, which reduces the leakage of metabolites (Lin *et al.*, 2016; Wang *et al.*, 2014).

Rapid filtration is also one of the effective methods to reduce metabolite leakage. In one study, 110 intracellular metabolites of *Saccharomyces cerevisiae*, four quenching methods, i.e., 60% (v/v) methanol, pure methanol (−40°C), boiling ethanol (75%, v/v), and rapid filtration, were compared (Kim *et al.*, 2013). The results showed that rapid filtration could greatly reduce the loss of metabolites.

Metabolite extraction is an important step in microbial metabolomics. The common methods of metabolite extraction are cold methanol, hot methanol, perchloric acid or alkali, chloroform-methanol mixture, and acetonitrile (Baidoo and Teixeira Benites, 2019; Mousavi *et al*., 2019). A comparison of seven methods for extracting *E. coli* metabolites by GC-MS analysis (Chakraborty *et al*., 2022) showed that ammonium and ethanol were effective methods for extracting *E. coli* metabolites, and 289 substances were detected. However, due to the diversity of metabolites, it is usually difficult to extract all intracellular metabolites by a single extraction method. Therefore, combining different methods is conducive to improving the extraction effect of metabolites.

Analysis and identification of metabolites

Analysis and identification of metabolites is the basis of metabolomics. Mass spectrometry and nuclear magnetic resonance are two main platforms for microbial metabolomics.

Mass spectrometry

Mass spectrometry is widely used in microbial metabolomics analysis due to its high specificity and sensitivity.

Gas chromatography-mass spectrometry (GC-MS) is a mature analytical platform and the earliest analytical method used in microbial metabolomics (Elmroth *et al*., 1992). GC-MS can simultaneously analyze hundreds of compounds (including organic acids, amino acids, carbohydrates, glycols, aromatic amines, and fatty acids) and possess a standard metabolite spectrum database (Migne *et al*., 2018; Wu *et al*., 2018). It can analyze metabolites quickly and accurately, but the samples need to be treated by derivatization. In recent years, many studies on microbial metabolomics based on GC-MS have been conducted (Baidoo, 2019; Baidoo and Teixeira Benites, 2019; Ponnusamy *et al*., 2013; Yang *et al*., 2014). Amino acid profiles of different filamentous fungi have been obtained using chloroformate derivatization. Fatty acids in yeast samples have been analyzed by microwave derivatization. The application of GC-MS technology (GC-GC-MS) possesses significantly improved the separation efficiency and sensitivity of complex samples and has been applied to microbial metabolomics. The volatile metabolites of *Pseudomonas aeruginosa* were analyzed by GC-GC-time of flight (TOF)-MS, and 28 new volatile compounds were identified, including alcohols, aldehydes, ketones, functional benzenes, and aromatic molecules (Adetunji *et al*., 2017; Bean *et al*., 2012; Depke *et al*., 2017).

Liquid chromatography-mass spectrometry (LC-MS) is another important platform for the analysis of unstable, non-volatile, non-polar compounds without derivatization of samples (Dodda *et al*., 2018; Zhao and Li, 2018). Hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS) is a high throughput intracellular metabolomics analysis technology that can analyze polar and non-polar metabolites simultaneously (Teleki and Takors, 2019). Its data acquisition and analysis speed are very fast. In the metabolic extracts of *Sinorhizobium meliloti*, 92.2% detectable polar and lipid metabolites were obtained by HILIC-MS (Fei *et al*., 2014). Ion chromatography-electrospray ionization mass spectrometry, as an effective platform for quantitative analysis of microbial metabolites, could be used for simultaneous quantitative analysis of several polar metabolites, such as nucleic acid, coenzyme A ester, glyconucleotide, and diphosphate (Jerome Jeyakumar *et al*., 2018; Reichert *et al*., 2018). Although LC-MS has been often applied in many studies, the study of microbial metabolomics using this method still encounters some problems. For example, the high salt concentration in the culture medium could inhibit the ionization efficiency of ESI, block pump, and ultimately affect the validity and repeatability of quantitative analysis.

Capillary electrophoresis-mass spectrometry (CE-MS) technology has the advantages of rapid analysis, less sample requirement, and less reagent consumption (Ortiz-Villanueva *et al*., 2017; Sato *et al*., 2017). In 2003, researchers used CE-MS to analyze 1692 metabolites extracted from *Bacillus subtilis*, 150 of them were identified, and different CE-MS platforms were used to analyze nucleosides, acetyl coenzyme A, cationic metabolites, and anionic metabolites, which provided a map for understanding the changes of metabolites during sporogenesis of *B. subtilis* (Soga *et al*., 2003). Pressure-assisted capillary electrophoresis-mass spectrometry (PACE-MS) was used to analyze the intracellular nucleoside and coenzyme A metabolites of *pykA* and *pykB* gene-deficient strains of *E. coli*, and led to the conclusion that *pykA* gene encodes for an enzyme (Soga *et al*., 2007).

Nuclear magnetic resonance (NMR)

NMR can rapidly and accurately analyze samples with high throughput and non-invasiveness (Araujo *et al*., 2019; Swann *et al*., 2017). It is an important analytical technique for identifying the structure of organic compounds, which could provide complete metabolic maps of biological tissues or body fluids under certain conditions (Kiselev and Novikov, 2018; Smolyanskaya *et al*., 2018). The study of microbial metabolomics has many broad application prospects (Almanza-Aguilera *et al*., 2017; Murovec *et al*., 2018). Using ^{1}H-NMR technology, the changes in metabolites during liquor fermentation under different effects of *Saccharomyces cerevisiae* could be monitored and the fermentation characteristics of yeast strains could be evaluated (Son *et al*., 2009). The intracellular metabolites of *Vibrio coralliilyticus* were analyzed by NMR at 27°C (highly toxic) and 24°C (non-toxic) incubation temperatures. Combined with principal component analysis (PCA) analysis, it was found that at different incubation temperatures, intracellular metabolites of *V. coralliilyticus* were significantly separated under PC1, PC2, and PC3 principal components, and with the increase in temperature, betaine decreased, while succinic acid and glutamic acid increased. However, due to the complex composition of microbial intracellular metabolites, including organic acids, hydrophobic substances, and complex natural products, changes in molecular concentration across several orders of magnitude (from pmol to mmol), while the sensitivity of NMR is low, it is difficult to simultaneously detect metabolites with different concentrations in biological systems (Barrilero *et al*., 2018). Hence, the application of NMR in microbial metabolomics is limited.

Data processing and analysis

Data processing and analysis are the key processes in metabolomics research, and the main steps of microbial

metabolomics data preprocessing and analysis are shown in Fig. 4. Consistent with metabolomics, data processing and analysis are briefly introduced in this review. It is necessary to pre-process the original data to eliminate the interference factors. Data processing generally includes noise elimination, baseline correction, peak alignment, binning, peak identification, normalization, and data scaling (Li *et al.*, 2018c; Liggi *et al.*, 2018; Montenegro-Burke *et al.*, 2017). At present, a large number of software can pre-process the original data obtained by MS into two-dimensional data tables, such as MZmine, XCMS, and METIDEA (Myers *et al.*, 2017). Many instrument companies have developed their proprietary software, such as Marker Lynx (Waters), Progenesis QI (Waters), Mass Profiler (Agilent), MarkerView (Applied Biosystems/MDS SCIEX), and ThermoFisher Science (Marzouki *et al.*, 2011; Tulipani *et al.*, 2011). Table 1 shows the main microbial metabolomics data processing and analysis software/websites. The pre-processed data need multivariate statistical analysis and bioinformatics analysis, such as PCA, and partial least squares discriminant analysis, to obtain effective information, identify biomarkers, metabolic pathways, etc. (Li *et al.*, 2018b). Metabolic pathway analysis is helpful to understand the interaction between metabolites and exploring gene expression data to complete functional genomics research.

However, microbial metabolomics still faces serious problems in signal processing and data analysis, which pose a great challenge in its research. To effectively eliminate the undesirable signal fluctuations introduced by environmental, instrumental, and biological factors, it is necessary to develop new methods for the optimization of metabolomics signaling systems and to tailor optimal data analysis strategies for different omics studies. In response to the above problems, some researchers have established a new method for metabolomics data processing based on machine learning and parallel computing for optimizing omics signal processing strategies (Fu *et al.*, 2021). This method can quickly optimize the best-performing omics data processing flow based on the metabolomics raw data given by the user by scanning the existing massive signal processing flow on a large scale. This approach enables data processing for "time-series" and "multi-taxonomic" metabolomics problems common to the field of microbiology.

Applications of microbial metabolomics

Microbial metabolomics is a discipline that combines bioinformatics and systematic microbiology. It is not only helpful in exploring the relationship between species but also a reliable analytical tool for the study of microbial evolution and development dynamics. As shown in Fig. 5, this review first summarized the research progress and application of microbial metabolomics from two aspects, probiotics and pathogenic bacteria. Because probiotics and pathogenic bacteria do not exist independently most of the time, we reviewed the research on the coexistence of probiotics and pathogenic bacteria. The research is subdivided into important microbial research fields closely related to human health, and the research progress and application of microbial metabolomics in the human gut, oral cavity, food and, nutrition-related microorganisms were reviewed. The recent progress and application of microbial metabolomics are discussed herein.

Probiotics

Lactic acid bacteria (LAB) are a generic term for bacteria that produce large amounts of lactic acid from fermentable carbohydrates (Liang *et al.*, 2020). In recent years, microbial metabolomics has made great progress and breakthroughs in the field of LAB research, such as strain screening and identification, metabolic pathway analysis, fermentation engineering, and beneficial effects (Kim *et al.*, 2017; Zhao *et al.*, 2016). The traditional classification of LAB is mainly based on morphological observation and biochemical experiments (Cai *et al.*, 1999; Makela *et al.*, 1992; Shaw *et al.*, 1985). With the development of molecular biology techniques, genotyping methods such as microbial genome sequencing, 16S rDNA sequence analysis, polymerase chain reaction (PCR) fingerprinting, and DNA hybridization technology have been widely used (Domingos-Lopes *et al.*, 2017; Ghodhbane *et al.*, 2016; Ramos *et al.*, 2018; Zhao *et al.*, 2015). However, the genotype and phenotype of some strains are inconsistent, resulting in different classification results. Metabolic profiling analysis could identify different strains by comparing the characteristic peaks of extracellular metabolites (Bachmann *et al.*, 2017; Filannino *et al.*, 2018). Microbial metabolomics has gradually become an effective, fast, and high throughput method. The extracellular metabolites of *Streptococcus mutans*, *Streptococcus haemophilus*, and *Lactobacillus acidophilus* were

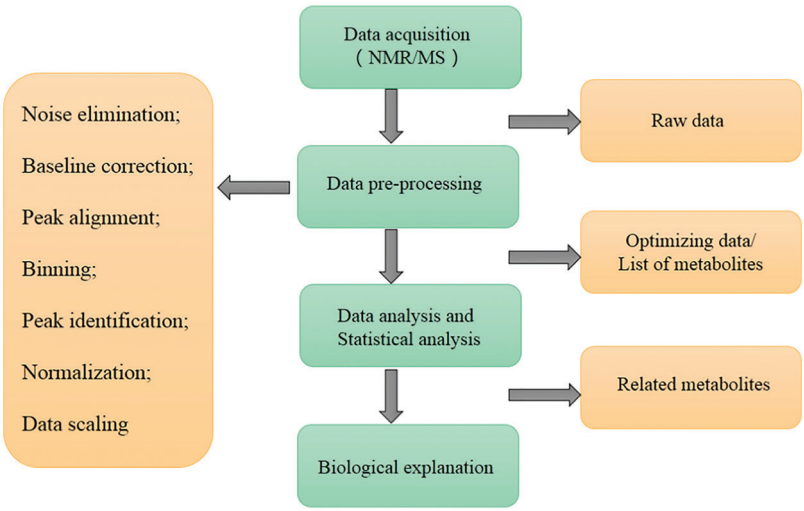

FIGURE 4. The main steps of microbial metabolomics data preprocessing and analysis. The main data processing procedures include: data acquisition, data pre-processing, data and statistical analysis, and biological explanation.

TABLE 1

Microbial metabolomics data processing and analysis software/websites

Name	Main application	Source	Reference/URL
ACD	Process NMR data	Advanced Chemistry Development, Inc. (ACD labs)	https://www.acdlabs.com/
AMIX tool-kit	Analysis of NMR, HPLC, and LS-MS data	Bruker TopSpin	https://www.bruker.com/en/products-and-solutions/mr/nmr-software/amix-outdated.html
Automics	NMR-based metabonomics	Softpedia	http://www.softpedia.com/get/Science-CAD/Automics.shtml
CCPN metabolomics	NMR-based metabolite studies	Collaborative Computational Project for NMR (CCPN)	http://www.ccpn.ac.uk/collaborations/metabolomics
CDK-Taverna	Workflow	GNU lesser general public license	
Chenomx NMR suite	Targeted profiling	Chenomx	http://www.chenomx.com/software
Chromaa	Alignment, chromatography-mass spectrometry	Free	
Chromaligner	Alignment of LC-MS data	Free	
COLMAR	NMR spectrum analysis	Florida State University	https://spin.ccic.osu.edu/index.php/colmar
DataChord spectrum miner	NMR processing and visualization	One Moon Scientific	http://www.onemoonscientific.com
KnowITAll metabolomics	Raw data processing to biomarker identification	BioRad Corporation	http://www.bio-rad.com
MetaboAnalyst	Web-based pipeline for metabolomic data processing	University of Alberta	http://www.metaboanalyst.ca
MetaboHunter	Identify individual metabolites in 1H-NMR	Natl Res Council of CA	https://www.mfitzp.com/metabohunter/
MetaboLab	Processing NMR data	University of Alberta	https://www.ludwiglab.org/software-development
MetaboMiner	Metabolite identification	University of Alberta	http://wishart.biology.ualberta.ca/metabominer
Metabonomic package	Statistical analysis of NMR data	GNU General Public License	
MetAlign	Importing many common formats of MS data	Free	
MetaXCMS	Importing XCMS output	Free	
Mnova	NMR and LC GC MS data processing	MestrelLab	http://mestrelab.com
MZmine 2	Data processing of MS data	Free	
Newton	Spectral analysis of multidimensional NMR data	NMRFAM	https://nmrfam.wisc.edu/newton/
NMRPipe	NMR spectroscopic data processing	National Institutes of Health (NIH)	http://spin.niddk.nih.gov/NMRPipe
NUTS	Processing and displaying NMR data	Acorn software	https://www.aiinmr.com/NUTS-Tips-Tricks-Deep-Dives
OpenMS	Raw data processing	Open source	http://open-ms.sourceforge.net/abou
PRIMe: SpinAssign	Metabolite identification	Platform for Institute of Physical and Chemical Research (RIKEN) Metabolomics	http://prime.psc.riken.jp/?action=standard_index
rNMR	NMR data visualizing and interpreting	NMRFAM	http://rnmr.nmrfam.wisc.edu
TopSpin	Processing NMR spectra	Bruker TopSpin	https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html?gclid
VnmrJ	Processing NMR data	Agilent	http://www.chem.agilent.com
XCMS	Processing LC-MS raw data	Free	
XCMS2	Importing tandem mass spectrometry	Free	

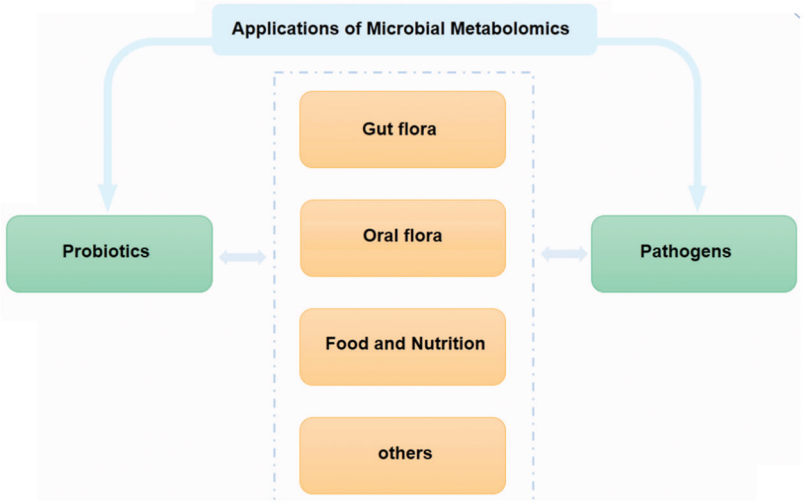

FIGURE 5. Main applications of probiotics and pathogenic bacteria microbial metabolomics. In recent years, research on microbial metabolomics has mainly focused on gut flora, oral flora, food, and nutrition.

studied by the ^{1}H-NMR microbial metabolomics method (Lee *et al.*, 2009). It was confirmed that the microbial metabolomics method could detect the differences among different strains and possess a good application prospect in microbial identification. Researchers compared the identification ability of LAB by PCR, 16S-amplified ribosomal DNA restriction analysis, and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and found that MALDI-TOF-MS had more advantages and accuracy at the species level (Doan *et al.*, 2012; Duskova *et al.*, 2012).

Microbial metabolomics is also widely used to monitor the changes in components and bacterial phase during fermentation and evaluate the sensory and nutritional quality of fermented food (Castro *et al.*, 2018; Pisano *et al.*, 2016; Renes *et al.*, 2019). High throughput sequencing and ^{1}H-NMR techniques were used to monitor the changes in microbial flora and metabolites during kimchi fermentation in South Korea (Jung *et al.*, 2012). The results showed that as a starter, *Leuconostoc mesenteroides* not only increased the proportion of *Leuconostoc* in the fermentation process but also decreased the proportion of *Lactobacillus*, shortened the fermentation time, and led to a higher production of organic acids and mannitol, which provided a direction for the regulation of kimchi fermentation. Meiju is a traditional fermented soybean paste in Korea. It is usually fermented by *Bacillus*, *Aspergillus*, and *Mucor*. Using UPLC-Q-TOF MS technology, the researchers revealed 22 markers in the Meiju fermentation process, including amino acids, small peptides, nucleic acids, ornithine circulating intermediates, and organic acids, and constructed the metabolic pathway of Meiju fermentation, which provided a theoretical reference for improving the nutrition and quality of Meiju products (Kang *et al.*, 2011). Therefore, metabolomics technology can directly detect the changes of components in LAB fermentation products, which provides an effective tool for process optimization and quality control (Zhao *et al.*, 2016).

In recent years, the effect of *Lactobacillus* on health has attracted extensive attention from researchers (Rezazadeh *et al.*, 2018; Zhang *et al.*, 2018). The effects of *Lactobacillus* on the intestinal tract by genomics, transcriptome, proteomics, and metabolomics have become a research hotspot (Awais *et al.*, 2018; Toshimitsu *et al.*, 2018). Microbial metabolomics can detect the changes in intestinal metabolites under the action of *Lactobacillus* and clarify the effects of probiotic metabolites on cytokine expression. The effects of probiotics on irritable bowel syndrome (IBS) mice were evaluated using ^{1}H-NMR-based microbial metabolomics techniques and clinical parameters (Hong *et al.*, 2011). IBS mice were found to have a potential energy imbalance (serum glucose) and liver dysfunction (serum tyrosine), and the symptoms improved by probiotic intake. The high-resolution magic angle spinning-NMR technique was used to evaluate the effect of probiotics on IBS patients (Hong *et al.*, 2011). The plasma concentration of glucose, tyrosine, and lactic acid in IBS patients who drank fermented milk containing *Lactobacillus* and *Bifidobacterium* for two weeks gradually became normal and thus provided a basis for exploring the mechanism of action of probiotics on IBS. Combined with GC-GC-TOF-MS and multivariate statistical analysis, it was found that *Lactobacillus rhamnosus GG* (LGs) could effectively regulate the structure and metabolism of intestinal flora in mice with alcoholic fatty liver and improve the health of the host (Shi *et al.*, 2015). Administration of LGGs increased the long-chain fatty acids in the intestine, decreased the fatty acid content in the liver, and increased the amino acid content in the liver (Castaneda-Gutierrez *et al.*, 2014; Ivanovic *et al.*, 2015; Ivanovic *et al.*, 2016). Therefore, microbial metabolomics is an effective tool to study the effects of probiotics on host health too. It could provide the metabolic response of host flora under the action of probiotics and lay a foundation for exploring the regulation mechanism.

Pathogens

In recent years, microbial metabolomics technology has been widely used in the research of pathogenic bacteria, which could analyze pathogenic bacteria in many aspects in detail (Akkerman et al., 2018; Fairley *et al.*, 2021). MS is the most commonly used microbial metabolomic analysis technology in the study of fungal plant pathogens (Sevastos *et al.*, 2018; Sirichokchatchawan *et al.*, 2018). It is widely used to analyze mutations in pathogens and to detect and screen secondary metabolites (Nielsen and Smedsgaard, 2003).

GC-MS analysis showed that the concentration of secondary metabolites in *Stagonospora nodorum* mutant

strains lacking the *Sch1* gene was 200 times higher than that of wild strains (Tan *et al*., 2009). ESI-MS/MS confirmed that the secondary metabolites were Alternaria phenol, which laid a theoretical foundation for elucidating the function and function of the *Sch1* gene. The researchers used GC-MS to analyze the metabolites related to the sporogenesis of wheat pathogen *Stagonospora nodorum*, and found that chitosan plays an important role in sporogenesis (Lowe *et al*., 2009). Microbial metabolomics has also been applied to diagnose diseases caused by pathogenic bacterial infections. GC-MS analysis of volatile organic compounds in feces reveals that metabolomic techniques could be used to distinguish diarrhea caused by different pathogens. Studies have revealed that furan could be detected in diarrhoeal feces of the patient infected by *Clostridium difficile*, while the presence of dodecanoate compounds in feces indicates that diarrhea is a rotavirus disease (Probert *et al*., 2004). Infections with *Rotavirus* and *Campylobacter* lead to the increase in terpenoids in feces. The decrease in dodecanoate compounds and the increase in amino compounds are the biomarkers of other enterovirus infections. While such studies provided a theoretical basis for rapid diagnosis of diarrhea etiology and a reference for the identification of different diarrhea pathogens, other studies have shown that some volatile substances are closely related to diseases caused by fungal infections.

Some researchers studied the metabolites of three different tissues (immature leaves, leaves, and sheaths) of ryegrass infected with endophytic fungi (*Neotyphodium lolii*) and ryegrass not infected with *N. lolii* and revealed the presence of mannitol and bolamine in the infected ryegrass by LC-MS (Cao *et al*., 2008; Li *et al*., 2018a; Wiewiora *et al*., 2015; Zhou *et al*., 2014). These results fully demonstrate that microbial metabolomics can identify bacterial species through metabolites secreted by pathogenic bacteria and also be an effective tool for the diagnosis of pathogenic bacterial infections.

Probiotics and pathogenic bacteria often coexist; therefore, it is necessary to study the field where the two coexist. The recent progress and application of microbial metabolomics in the important microbial research fields closely related to human health are discussed in the following sections.

Gut flora

In the human body, the number of microorganisms is much larger than that of cells, and gut flora is one of the key points in systematic biology and metabolomics (Geng *et al*., 2018; Ke *et al*., 2018a, 2018b; Si *et al*., 2018; Liu *et al*., 2022). The functions and metabolism of microorganisms are closely related to the health and disease of the host (Song *et al*., 2018; Suzuki-Iwashima *et al*., 2020; Xing *et al*., 2018). They can prevent the infection of pathogens and provide energy for the host through their own metabolism, enhance the immunity of the host, and regulate the metabolic phenotype through their interaction with the host, and so on (Bhatnagar, 2015; Jain *et al*., 2012; Wang *et al*., 2011; Na and Lim, 2022).

The metabolic interactions between gut flora and host in mice models were studied by various techniques, including ^{1}H-NMR analysis of metabolic profiles of the liver, plasma, urine, and ileum contents, LC-MS targeting analysis of bile acids, and GC technique detecting fatty acid fingerprints in the cecum (Martin *et al*., 2007). The concentration of bile acid was higher in ileum and triglyceride in the liver of sterile mice colonized with infant gut flora than that of normal mice, while the level of plasma lipoprotein was lower. These data showed that gut flora could regulate intestinal absorption and energy storage and acquisition.

Microbial metabolomics technology based on MS was used to study the plasma of aseptic mice and normal mice (Wikoff *et al*., 2009), and the results revealed that gut flora possessed significant effects on the plasma metabolites of mammals. For example, the antioxidant 3-indolepropionic acid only exists in the plasma of normal mice, but *Clostridium* was colonized in aseptic mice. After um sporogenes, 3-indolepropionic acid was also detected in plasma, indicating that the production of 3-indolepropionic acid in the host was influenced by gut flora. The water-soluble fecal metabolites of humans, mice, and rats were compared by ^{1}H-NMR (Ganda Mall *et al*., 2018; Saric *et al*., 2008). These three metabolites were found to have unique fecal metabolite profiles. For example, beta-alanine was detected only in rat feces, while glycerol and malonate were found to be unique to human feces. It was thus confirmed that different gastrointestinal microorganisms have significant effects on fecal metabolites. Therefore, microbial metabolomics technology could directly detect the changes in host metabolic phenotype under the changes in gut flora, which provides a new research strategy and method for further elucidating the interaction mechanism between gut flora and the human body (Goodwin *et al*., 2015; Lyte *et al*., 2019) (Fig. 6).

Normally, gut flora structure plays an important role in the prevention and control of diseases, but gut flora imbalance and changes in microbial biodiversity lead to a series of adverse effects on the host, such as the occurrence of various gastrointestinal diseases, metabolic diseases, and immune diseases (Jain *et al*., 2012; Nagai, 2015; Wang *et al*., 2017b). Many researchers have used microbial metabolomics technology to study gut flora and host metabolites to explore the effects of gut flora on host health and disease and have achieved various important results (Geng *et al*., 2018; He *et al*., 2018; Ke *et al*., 2018b). GC-MS and pyrosequencing were used to study the changes in volatile metabolites and gut flora structure in stool samples of obese patients with non-alcoholic fatty liver (Raman *et al*., 2013). The results showed that the increase in esters in the stool of non-alcoholic fatty liver obesity patients was related to the changes in gut flora structure.

Researchers used GC-MS technology to study the metabolomics of fecal volatile organic compounds in IBS, Crohn's disease, ulcerative colitis patients, and healthy people (Ahmed *et al*., 2013; Furnari *et al*., 2016; Rossi *et al*., 2018). The results showed that IBS occurred in patients harboring short-chain fatty acids, cyclic acetic acid, and acetic acid. Microbial metabolomics and aseptic mouse experiments showed that phosphatidylcholine metabolism in gut flora can promote the occurrence of cardiovascular diseases, and gut flora plays an important role in the

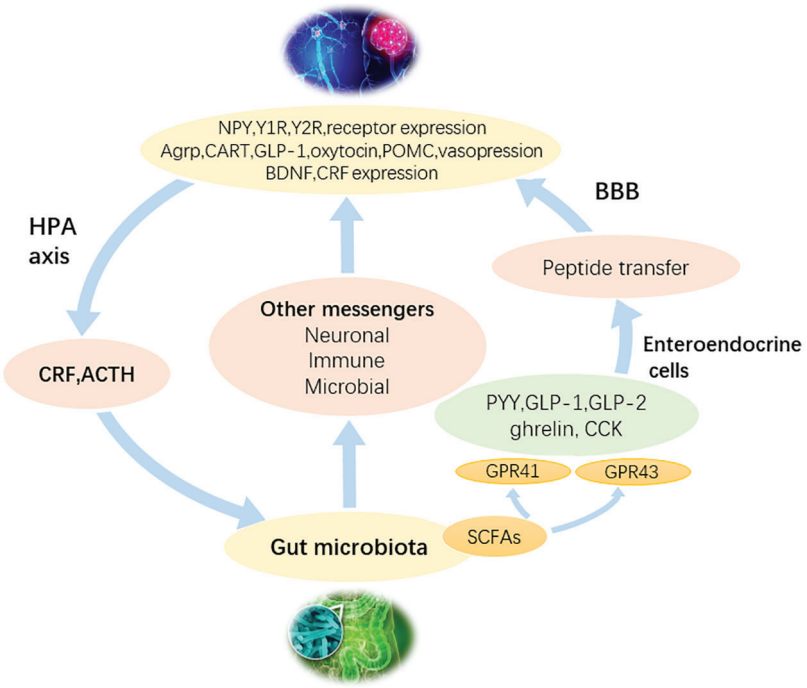

FIGURE 6. A flowchart to depict the mechanism of the effect of gut flora on the brain. Gut flora could interact with the brain through a variety of signal pathways.

production of trimethylamine and the formation of foam cells (Bhatnagar, 2015). Therefore, it is essential to analyze gut flora and host co-metabolism by microbial metabolomics technology to reveal the metabolic function of gut flora and their effects on host health and disease.

Oral flora

Recently, microbial metabolomics technology has gradually penetrated the field of stomatology (Shakhatreh *et al.*, 2018); it has been reported in the study of microorganisms, caries, periodontal diseases, cleft lip, palate, and oral tumors. Most of them are used to analyze the metabolites of cariogenic bacteria and periodontal pathogens in the oral cavity. The oral cavity harbors more than 700 species of prokaryotes (Mager *et al.*, 2003). Some bacteria could cause oral infectious diseases, such as dental caries, periodontitis, pulp disease, alveolar osteitis, and glossitis (Burczynska *et al.*, 2017; Dong *et al.*, 2018; Krzyzek and Gosciniak, 2018; Xu *et al.*, 2015). Different oral structures and tissues are colonized by different microbial communities. Oral microbial identification is very important for the etiology, treatment, and prevention of infectious diseases such as dental caries, and periodontal diseases (Lira-Junior *et al.*, 2018; Mombelli, 2018; Rafiei *et al.*, 2018).

When using microbial metabolomics to identify *Streptococcus mutans* and *Actinomyces viscosus*, the researchers found that PCA could classify and identify the two species of bacteria, and the one-dimensional spectrum time of magnetic resonance samples was only 5 min, which was convenient and fast (Kunze *et al.*, 2010; Ribeiro *et al.*, 2017; Salman *et al.*, 2018). The spectra of extracellular metabolites of *Actinomyces tunica*, *Actinomyces neisseriae*, *Streptococcus sanguis*, *Streptococcus cousins*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Clostridium nucleatum*, *Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces viscosus*, and *Candida* in a stable phase were compared by the same method (Hetrodt *et al.*, 2018; Sanz *et al.*, 2017). The difference between different strains could be well detected. Microbial metabolomics analysis of *Streptococcus* and *Actinomycetes* showed that the metabolic pathways of these two microorganisms were different. Researchers applied microbial metabolomics to the *in vivo* study of dental plaque biofilm and established dental plaque biofilm metabolomics (Keijser *et al.*, 2018). When analyzing the effects of xylitol and fluoride on the metabolomics of plaque biofilm, fluoride was found to inhibit the glycolysis pathway of supragingival plaque but had no effect on the acid products of xylitol and the metabolic profile of supragingival plaque (Takahashi and Washio, 2011).

The analysis of the whole metabolome of the early oral cavity showed that the saliva metabolites of children with caries in deciduous dentition, mixed dentition, and permanent dentition were different. Compared with the normal group, the contents of lactic acid, acetic acid, and butyric acid increased in the infected group. The composition of metabolites in the saliva of normal children was similar, although their oral hygiene habits, socioeconomic status, and diet were different (Fidalgo *et al.*, 2013). In addition, the salivary metabolites of children with dental caries after 3 months of composite resin repair showed a significant decrease in propionic acid, acetic acid, butyric acid, and oligosaccharide content, accompanied by a decrease in the culture level of *Streptococcus mutans* and *Lactobacillus* (Fidalgo *et al.*, 2015). Salivary metabolites analysis in children with caries showed a prominent role of the metabolites involved in the metabolic pathway of arginine proline and the acid-base balance connecting arginine and alkali production (Edlund *et al.*, 2015). Microbial metabolites in adult supragingival plaque were also analyzed in terms of the Embden-Meyerhof-Parnas pathway, pentose phosphate path way, and tricarboxylic acid cycle (Takahashi *et al.*, 2010), and the main metabolic

pathway was found to be the supragingival plaque. It involves all the carbohydrate metabolites except erythrose-4-phosphate in the pentose phosphate pathway. After rinsing with glucose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, dihydroxy acetone phosphate, pyruvate, and pentose phosphate pathways in the glycolysis pathway were determined. Further, 6-phosphogluconate, ribu-lose-5-phosphate, sedoheptu-lose-7-phosphate and acetyl CoA increased. Meanwhile, 3-phosphoglycerate and phosphoenolpyruvate, succinate, and malate in the tricarboxylic acid cycle decreased in the glycolysis pathway. These changes in pathways and metabolites observed in supragingival plaque are similar to those observed in *Streptococcus* and *Actinomycetes*. The composition of dental caries-related microbial communities and their relationship between them are diverse and huge. Microbial metabonomics possesses a significant space in the study of oral flora.

Food and nutrition

In recent years, food safety has received much attention. Pathogens, toxins, and by-products produced by microbial degradation of food are closely related to food safety (Putri *et al*., 2022). Therefore, monitoring these related metabolites is very important for food safety. Microbial metabolomics provides a new strategy for food safety assessment. It has been successfully applied to the detection of toxic substances in food, such as the fingerprint of volatile metabolites related to specific microbial contamination using GC-MS technology and the detection of microbial toxins in food by LC-MS and NMR technology (Qian *et al*., 2015). Researchers used GC-MS to detect toxins such as zearalenone produced by *Fusarium* spp. in edible vegetable oils. Studies used LC-MS/MS to detect and quantitatively analyze 23 mycotoxins in different sorghum varieties (Forero-Reyes *et al*., 2018; Njumbe Ediage *et al*., 2015).

Microbial metabolomics has also been used to assess the effects of nutrient deficiency and excess on metabolic balance, accurately monitor the effects of diet on the body, and reduce the interference of confounding factors such as age, sex, physiological status, and lifestyle (Fu and Cui, 2017). Techniques including CE, RP/UPLC and HILIC/UPLC-TOF-MS revealed the significant effect of dietary polyphenols on the anti-proliferation of human colon cancer HT29 cells (Ibanez *et al*., 2012). Non-targeted metabolic analysis showed that the ratio of glutathione, an antioxidant, increased after the treatment of polyphenols, while the expression of polyamines, which maintained cell proliferation and regulated gene expression, inhibited cell growth, and provides a theoretical basis for the prevention and treatment of colon cancer.

Based on the microbial metabolomics technique of NMR, researchers monitored the urine metabolic profiles of normal puppies and dietary restricted puppies (Wang *et al*., 2007). The results showed that dietary restriction could change the intestinal microbial activity of puppies, which was mainly manifested in the increased concentration of aromatic metabolites, creatine/creatinine, and acetic acid compounds in the urine of dietary restricted puppies. These compounds were closely related to gut flora. Analysis of the ^{1}H-NMR metabolic profiles of piglet urine confirmed that different weaning diets produced different metabolic phenotypes, which led to changes in gut flora, and host metabolites. Studies have also found that intestinal mucosal products associated with lgA and lgM were produced by *Bifidobacterium* NCC2818 ingestion during the weaning diet, which provided a new research strategy for evaluating mucosal immune status. These findings fully demonstrate that microbial metabolomics can quickly and effectively make a comprehensive assessment of animal health, disease prediction and diagnosis and further understand the interaction between nutrition and metabolism in the body.

Discussion

In summary, microbial metabolomics has been widely applied in microbiology research and provides a comprehensive and systematic analytical technology for microbiology research. Metabolic products in microbial cells are of great value in the analysis of cellular metabolic processes and pathogenic processes, as well as the effects of drugs or the environment on bacteria. Bacterial metabolites consist of complex mixed metabolites and are associated with a large number of metabolic pathways and signal transduction pathways. This high degree of correlation may be more meaningful than the original disturbances such as inhibition, inactivation or down-regulation of proteins. It is easier to observe the changes in metabolites.

To understand the physiology of bacteria or other microorganisms and obtain reliable results, it is necessary to identify and establish at least two reference groups for comparative analysis, such as wild type and mutant type, drug resistance type and susceptibility type, and nutritional enrichment and nutritional deficiency. Only when reference conditions are established, bacteria could be exposed to any experimental variables such as drug treatment, environmental stimuli, or gene knockout to determine any possible similarities in reference metabolites. With the continuous improvement in sample preparation methods and the rapid development of analytical techniques, great progress has been made in microbial metabolomics research. It could be used for the study of biomarkers in the microbial metabolism process and provide a comprehensive and effective evaluation method for monitoring the fermentation process, safety detection, and pathogenic bacterial infection diagnosis. It could be used to study the metabolic mechanism of intestinal flora and host and provide theoretical basis for the prevention and treatment of metabolic diseases.

Some problems in microbial metabolomics of probiotics and pathogens still need to be solved. First, there is a lack of effective methods for quenching and extracting metabolites. Microbial cell metabolism is very sensitive to changes in the surrounding environment, and both the measurement and sample preparation process could affect metabolomics. Microbial metabolomics lacks good methods for rapid inactivation of metabolic activity, comprehensive extraction of metabolites, and analysis of specific metabolites. Second, although many microbial metabolomics databases have emerged, they are limited to specific microbial populations. At present, microbial metabolomics and databases are mainly confined to specific microorganisms, mainly yeast,

and *E. coli*, and lack integrated databases containing different microbial metabolic data, especially for bacteria and fungi. Third, microbial metabolomics still faces problems of the signal processing and data analysis, which poses a great challenge. It is necessary to develop new methods for the optimization of metabolomics signaling systems and to tailor strategies for optimal data analysis for different omics studies. Artificial intelligence and machine learning will be the trend to solve the problem of microbial metabolomics. Fourth, the study of microbial metabolomics pays much attention to metabolites rather than their sources. For example, although the chemical composition and structure of glucose from the host and microbial metabolism are identical, their biological significance and metabolic pathways involved make their regulation different. The study of microbial metabolism and host changes is of great significance to the study of the microbial-host relationship.

Compared with the application of metabolomics in drug research, disease diagnosis, and plant metabolomics, microbial metabolomics is still in its infancy. However, there are many advantages of microbial metabolomics research, such as a simple microbial system, rich genetic data, and a comprehensive understanding of microbial physiological characteristics. Concurrently, the integration of microbial metabolomics with genomics, transcriptome, and proteomics could help understand organisms more systematically by studying metabolic pathways, regulatory responses, and homeostasis mechanisms *in vivo*. In summary, microbial metabolomics, as a new research field with rapid development, is an important component and technical platform of systems biology, which promotes the development of systems microbiology and artificial intelligence.

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