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REVIEW

Fundamentals and Analytical Strategies for Metabolomics Workflow: *An Overview and Microbial Applications*

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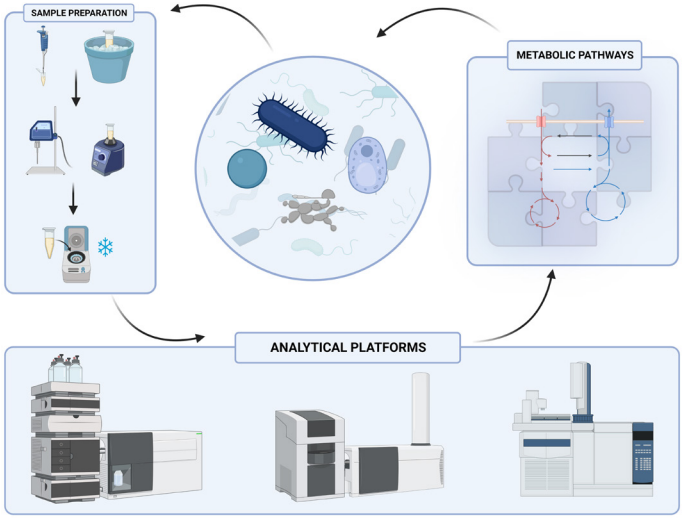

Metabolomics has become a prominent area within the omics sciences and allows an understanding of complex biological systems. Among several areas of knowledge, the study of microorganisms (microbial metabolomics) has received attention. Due to the many species of microorganisms and their high metabolic complexity, many challenges are involved in metabolomics workflow. Careful experimental design and execution of the experiments will provide reliable results, allowing correct biological interpretation. This review presents the fundamentals of metabolomics and workflow, focusing on the description of the steps and analytical strategies applied to microbial sample preparation, highlighting the current challenges in sample handling. In addition, the state of the art of analytical technologies based on separation techniques hyphenated to mass spectrometry and applications in microbial metabolomics are presented.

Keywords: sample preparation, mass spectrometry, chromatography, capillary electrophoresis, microbial metabolomics

INTRODUCTION

Metabolomics is a post-genomic approach used to determine alterations in metabolite levels, which are the end product of metabolism. The term metabolomics appeared for the first time in 2001,[1] and since then, it has been used in works that aim to understand biological processes. The determination of biochemical changes is performed by comparing sample groups with different environmental influences, genetics, or external interventions (drug treatment, diet, etc.). Therefore, this bioanalytical strategy combines careful sample preparation, high-throughput analytical instruments, bioinformatics, and chemometrics. Thus, metabolomics presents itself as a powerful phenotyping tool.[2,3]

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The analysis strategies involve two complementary approaches, named untargeted and targeted metabolomics. Untargeted metabolomics is a hypothesis-generated approach in which as many as possible metabolites are semi-quantitatively determined to obtain global information about the biological organism. In contrast, targeted studies, a hypothesis-driven strategy, quantify specific metabolites or chemical classes selected according to the prior knowledge of the organism under investigation.[4]

The applications of metabolomics permeate different areas of knowledge, from clinical studies using biological fluids (to understanding diseases for diagnostic and prognostic purposes),[5-8] in the analysis of plants,[9,10] foods,[11] and even cellular metabolism focused on different microorganisms,[12-15] denominated microbial metabolomics. This review presents some fundamentals and discussion of analytical strategies employed in metabolomics, emphasizing the technical aspects and challenges applied to microbial metabolomics.

MICROBIAL METABOLOMICS

Metabolomics of microorganisms (MO) is one of the growing areas of study within the omics sciences, given its wide application in biotechnology and microbiology fields. This is observed by the linear increase of published works in the last years (Figure 1). Such publications comprise mainly studies on microbiota (gut microbiota), given the physiological effects on health, disease prevention, and immune system improvement due to the colonization of microorganisms in the human body. Other applications include understanding cellular metabolism, identifying MO species, and regulatory metabolic pathways.[16]

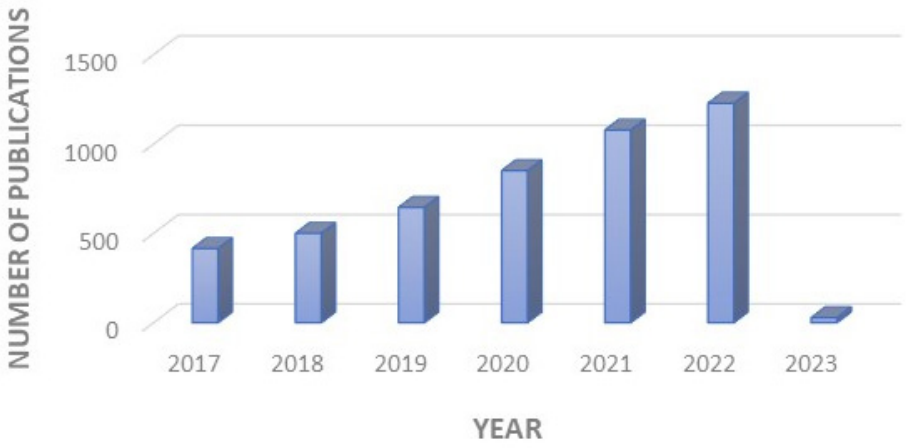

Figure 1. Literature inspection related to “microbial metabolomics” in the title, abstract, or keywords from 2017 to 2022 on PubMed database.

Challenges in microbial metabolomics encompass the difficulty of developing standardized methodologies for sample preparation. The differentiation between exometabolome (extracellular metabolites) and endometabolome (intracellular metabolites) is laborious and time-consuming, making it necessary to optimize the extraction step due to the large number of existing species and different cell membrane compositions. Another bottleneck in such investigations is the extensive amount of metabolites present in these organisms. This makes it difficult the metabolite assignment, being necessary to classify them as “unknown metabolites”, and important biological information is lost.[16,17]

METABOLOMICS WORKFLOW

The metabolomics workflow involves several steps, from experimental design to the determination of altered metabolites and biological interpretation (Figure 2). Biological problem definition and decisions about the type and number of samples, study groups, and sample collection are the initial steps of this workflow. An appropriate experimental design is crucial and will define the analytical procedures applied,

especially considering the type of approach (if targeted or untargeted metabolomics) and the analytical platforms used. The following steps include sample preparation and data acquisition by different analytical platforms, whose theoretical and practical aspects will be discussed in the following sections of this review.

Metabolomics raw data are complex and require dedicated software for processing and generating the data matrix (2D). Methods and advances in metabolomics data processing have been recently revised.[18,19] Altogether, processing steps involve the detection of analytical signals, background removal, alignment, clustering, and normalization. Different tools, from instrumental vendors to open-access software, are applied for this purpose.[4] It is important to emphasize the need for extra attention in processing methods, to maintain the integrity of biological information and accuracy in interpreting metabolic changes. Subsequently, chemometric methods and univariate statistical analysis are used to find differentiation between the groups under investigation. Several review articles bring fundamentals and methods of statistical analysis commonly applied to metabolomics studies.[20-22]

Metabolite assignation is another critical step in the metabolomics workflow and has been considered challenging, requiring sophisticated instrumentation, authentic analytical standards, and database inspection. The assignation of analytical signals can be performed in two ways: by annotation or identification. Metabolite annotation (also called putative annotation) is a tentative assignment based on the search of the mass (*m/z*) in different libraries or databases (such as METLIN, HMDB, LipidMaps, PubChem, etc.). On the other hand, metabolite identification is more accurate, requiring experiments based on tandem mass spectrometry (MS/MS or MS^n) analysis followed by comparing spectral fragmentation patterns and retention time with authentic analytical standards are performed.[23] Ultimately, the metabolomics workflow encompasses the raising of hypotheses and the elucidation of the biological question. Thus, pathway enrichment analyses have been applied to help understand the metabolic alterations.[24] Finally, the results can be validated using new cohorts and performing novel metabolomics experiments.

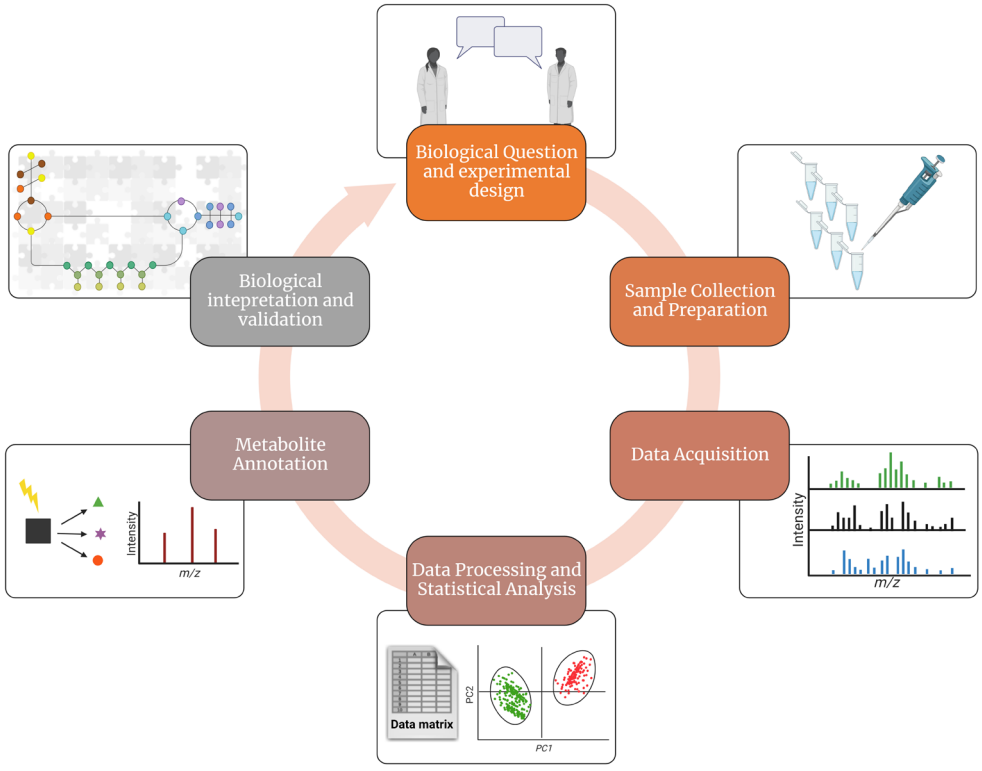

Figure 2. General metabolomics workflow. Created with “BioRender.com”.

Sample Preparation

Sample preparation is a crucial step in the workflow and reflects the success of a metabolomics study. Rapid conversion of metabolites (generally 1-2 s), cell degradation, metabolite leakage, extraction efficiency (in the face of large chemical variability), and reproducibility are considered the major challenges in this step.[16,25] Regarding cellular analysis, in addition to the basic quenching and extraction steps, other factors must be considered, such as preparation of the culture medium, number of passages, and sample harvesting. Therefore, every experiment must be carefully designed. In the case of microorganisms, due to the different growth conditions and cell wall composition, it is recommended to optimize all stages, from cell inoculation and maintenance to metabolite extraction.[26] Furthermore, accurate results are achieved after an appropriate choice of procedures, depending on the metabolomics approach (targeted or untargeted) and analytical platform.[27]

The reproducibility of cell growth is obtained with the establishment of controlled conditions, such as pH, light, temperature, medium composition, and dissolved gases. Usually, MOs are grown in bioreactors, which can operate under fixed nutrient conditions (fed-batch) or with a renewal of culture medium and biomass (chemostat).[28] Sample collection should be rapid and avoid metabolite turnover rates. In order to perform that, the inactivation of enzymes by quenching methods is necessary.[29]

Metabolic quenching is a continuous process within sample preparation in which enzymes are rapidly deactivated, and cells must maintain their integrity.[17,29] Different protocols are found in the literature[30] and include exposing samples to extreme temperature and pH conditions. Cold organic solvents (methanol or acetonitrile, for example), pure or in saline mixtures, have been applied for quenching of several MO species, such as *Pichia pastoris,*[15] *Lactobacillus plantarum,*[31] and *Corynebacterium glutamicum.*[32] Other methods include liquid nitrogen[33,34] and fast filtration.[35,36] However, due to the formation of ice crystals, the promotion of metabolite leakage, and fast turnover rates of some classes of metabolites these methods are less used. Alternative quenching procedures involve the application of a glycerol-saline[37] or acid/alkaline solutions.[29,30] Often, these methods culminate in metabolite leakage by the high susceptibility of the cell membrane, causing significant loss of metabolic information and interference by the glycerol solution.[30] Thus, there is no consensus regarding the best quenching protocol, but it is known that it is organism-dependent. The application of the quenching step must take into account the effectiveness of the process and reproducibility. For this reason, methods must be continuously optimized for each organism under study.

A complete understanding of the metabolism of microorganisms is obtained after analyzing the extracellular and intracellular portions. In order to perform that, pellet separation from the culture medium is carried out by centrifugation or fast filtration.[25] For intracellular analysis, however, a washing step is necessary before extracting the metabolites to completely remove interferents from the culture medium or compounds secreted by the MO. Usually, NaCl saline solutions[38,39] or phosphate-buffered saline solution (PBS)[12,40,41] are applied. It is important to highlight that these solutions must be used at low temperatures, to maintain the inactivation of the enzymes.

Intracellular extraction combines membrane disruption methods with the extractor solvent.[27] Cell lysis can be performed mechanically, with instruments such as sonicators, tissuelyzers, vibration mills, etc., or even by freezing-thawing cycles.[42] Methanol, ethanol, acetonitrile, and chloroform (pure and mixtures) have been frequently employed to access the endometabolome in different MO.[30] Aqueous methanol and acetonitrile solutions have been the most applied in microbial metabolomics. Such extractants combined with ultrasound bath and vortex mixing were used to extract intracellular metabolites from bacteria[12,13,36] and fungus.[15,35] The choice of the type of lysis and solvent extractor is closely related to the analytical technique used and, consequently, metabolite physicochemical properties. In addition, the ease of permeability of the cellular membrane is also a factor that influences lysis.[27] It is known that gram-positive bacteria, for example, have high concentrations of peptidoglycan in their membranes, providing greater mechanical resistance compared to gram-negative, which are essentially composed of lipopolysaccharides.[43] In this sense, several works focus on optimizing intracellular extraction,[32,36,44] highlighting the importance of systematic evaluations in the study of MO.

Sample preparation for extracellular metabolomics compared to intracellular is quite simple. The quenching step is not necessary, since there is no enzymatic activity in the culture medium.[27,45] Extracellular metabolites come from cellular secretion processes, the composition of the culture medium, or even cell leakage. Thus, any changes in the exometabolome composition result from external factors associated with the cell growth environment (such as pH, temperature, and nutrients).[28,46] Culture medium supernatants are usually subjected to simple dilution, deproteinization, desalting, preconcentration, or evaporation.[17] Such procedures are carried out in order to remove interferences such as sugars, proteins, salts, and lipids present in high concentrations in the culture medium. These species can cause malfunctions in the analytical instruments through the precipitation and consequent obstruction of the chromatographic system. In addition, problems with ionization suppression and sensitivity decrease are frequently observed.[27,46] Different protein precipitation and salt removal methods require large solution volumes, resulting in low recovery of metabolites.[46] Alternative methods for extraction and preconcentration, such as solid phase extraction (SPE) and solid phase microextraction (SPME), have shown good results when applied to microbial metabolomics. Despite the great advantage of using a reduced volume of toxic solvents and still determining low concentrations of analytes (parts per trillion, ppt to parts per million, ppm), such methods are expensive and selective, being applied more frequently in targeted studies.[46,47]

This topic provides an overview and challenges of the methods applied to sample preparation for microbial metabolomics. More details can be found in recently published review articles[27] focusing on intracellular,[30] extracellular,[46] and bacterial metabolomics.[25] Due to the increased interest in the study of microorganisms and the lack of universal sample preparation methods, optimizations, and the establishment of protocols are encouraged in order to improve extraction efficiency and, consequently, the generation of reliable and reproducible results.

Analytical Platforms

The most applied technologies in metabolomics studies are based on mass spectrometry (MS) and nuclear magnetic resonance (NMR). The former is often coupled with chromatographic techniques (liquid, LC, and gas chromatography, GC) or capillary electrophoresis (CE). Such couplings provide better sensitivity and selectivity in detecting the thousands of metabolites that comprise the metabolome.[48] Ye and co-workers[17] recently reviewed the technological novelties applied to microbial metabolomics. A careful survey of the literature showed the growing application of MS and hyphenated platforms (GC-MS, LC-MS, and CE-MS), comprising more than 57% of publications versus 31% of NMR in the last 22 years. Table I presents an overview comparison of the advantages and disadvantages of separation techniques hyphenated to mass spectrometry (LC-MS, GC-MS, and CE-MS) applied to metabolomics studies. Another highlight is the use of more than one analytical platform, especially in the last ten years, comprising around 11% of the works. It is known that no analytical technique is capable of covering the entire metabolome due to the great diversity of metabolites with variable concentration ranges. In this sense, the use of more than one platform has been recommended in the study of MO. Thus, given the relevance and scope of separation techniques coupled to MS, the general aspects and some applications in microbial metabolomics are presented in the following subsections.

Table I. Advantages and disadvantages of the separation techniques hyphenated to mass spectrometry applied to metabolomics studies

Analytical Platform	Advantages	Disadvantages
GC-MS	✓ Good repeatability ✓ Robustness ✓ High resolution ✓ High detectability ✓ Detection of volatile compounds ✓ Available libraries/databases	✓ Time-consuming sample preparation ✓ Non-derivatized samples (outliers) ✓ Moderate metabolic coverage ✓ Difficult to identify novel compounds
LC-MS	✓ Versatility ✓ Good repeatability ✓ High resolution ✓ Wide metabolic coverage	✓ Identification requires analytical standards and MS/MS analysis ✓ Different columns and separation modes to cover metabolome ✓ Lower reproducibility than GC-MS
CE-MS	✓ Low sample volume ✓ Simple sample pretreatment ✓ High resolution	✓ Identification requires analytical standards and MS/MS analysis ✓ Low repeatability than LC-MS and GC-MS ✓ Low sensitivity (dilution by sheath liquid)

GC-MS

Gas Chromatography coupled to Mass Spectrometry (GC-MS) is one of the analytical techniques most applied to metabolomics studies and has been gaining prominence in microbiology.[48] This technique has been used to characterize the volatile metabolome since it has excellent resolving power in the separation of non-polar and volatile or chemically volatilizable metabolites.[49] Overall, analyte separation is conducted in bore fused-silica capillaries filled with non-polar stationary phases, such as 5%(diphenyl)-polydimethylsiloxane (PDMS).[48] Recently, the use of 2D GCxGC, using columns with more polar phases (with higher % of diphenyl-PDMS and cyanopropylphenyl-PDMS, for example), has increased the selectivity and coverage of the metabolome by improving the separation of isomers.[50] In microbial metabolomics, GCxGC was applied to analyze the volatile exometabolome of *Candida albicans*, *Candida tropicalis*, and *Candida glabrata*. The methodology provided an annotation of 126 metabolites, including acids, alcohols, ketones, terpenes, aldehydes, among others. This chemical characterization of species can provide insights into clinical diagnosis, guiding pharmacological interventions.[14] Another interesting work used two-dimensional analysis to assess metabolic changes mediated by ozone stress in *Cobetia marina*, model bacteria for biofouling. The untargeted analysis using the non-polar and mid-polar chromatography column allowed the observation of a reduction in the content of fatty acids and amino acids by increasing ozone concentration.[51]

The GC-MS coupling provides fast, sensitive, and selective analyses allowing the separation and quantification of hundreds of metabolites, due to the high sensitivity and resolving power, especially when using time of flight (ToF) analyzers.[52] In addition to these advantages, the almost universal use of electron ionization (EI), typically operating at -70 eV, causes the high energy supplied to molecules to produce ion fragments. The highly reproducible fragmentation pattern, characteristic of each molecule, allows the application of several spectral libraries (NIST or online databases) for metabolite assignment and annotation. In order to improve the level of confidence in metabolite identification by combining information on retention time and mass spectra, it is recommended to build own libraries using authentic analytical standards or by co-injection of labeled compounds.[48,50]

Metabolome coverage by GC-MS is limited to the analysis of volatiles. Thus, in order to improve the detectability and metabolite volatility, and stability at working temperatures, chemical derivatization reactions are required.[16,48] Derivatization aims to make the metabolites volatile and thermally stable. Several methods have been applied in metabolomics studies, but the two-step oximation and silylation are the most frequently used. This method consists primarily of adding O-methoxyamine to completely dried extracts, aiming to protect carbonyl groups and prevent the cyclization of reducing sugars. The volatility reduction by the following silylation step is performed by the replacement of active hydrogens by trimethylsilylate (TMS) group.[52] Despite using well-established and extensively studied methods, this additional sample preparation step is often performed manually. It is time-consuming and influences analytical performance, especially by the loss of metabolites due to its volatility.[25] Online derivatization using automated systems has been used to improve the reproducibility of generated data. This robotic system was applied in the derivatization of intracellular metabolites of *Candida albicans*, reducing the total reaction time to a few hours.[33]

An automated system that analyzes volatile compounds without requiring derivatization is based on solid-phase microextraction combined with headspace (HS-SPME). In this solvent-free method, the analytes are collected in the headspace after volatilization in a highly reproducible way and short time.[30] HS-SPME has been successfully applied in the search for volatile biomarkers discriminating between seven different species of mycobacteria responsible for causing diseases such as leprosy and tuberculosis.[53] Another study sought the determination of volatile organic compounds (VOC) by HS-SPME-GC-MS in yeast strains for the biocontrol of post-harvest diseases caused by contamination by harmful mycotoxins.[54]

LC-MS

Hyphenated liquid chromatography with mass spectrometry (LC-MS) has been the most applied analytical platform in metabolomics studies.[55] The chromatographic separation is based on the partition difference between the analyte (metabolite) and the stationary and mobile phases, according to their physicochemical characteristics of polarity, charge, and size.[56] Gradient elution is often used to analyze complex samples such as cells and biological fluids. This elution improves the resolution between chemical species and is especially interesting for untargeted approaches.

One of the main advantages of LC is its versatility in terms of the metabolites classes that this technique can separate. This is achieved due to different separation modes. Metabolomics studies are generally restricted to reverse-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) analyses. RPLC uses a non-polar separation column and a more polar mobile phase, facilitating the separation of non-polar and moderately polar metabolites. HILIC, on the other hand, presents a polar stationary phase and a mobile phase containing an initial mixture of organic solvent and low % water, with a progressive increase in the aqueous portion throughout the chromatographic run. Thus, HILIC is interesting for separating highly polar substances, complementing RPLC.[57,58] Recent advances in this separation technique include the development of columns with smaller particle sizes, in addition to porous (sub-3 µm and sub-2 µm) or fused-core particles for faster and more efficient separation. High-pressure liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) instruments, the latter capable of withstanding pressures greater than 10,000 psi, have been widely used in metabolomics.[56] Interesting compilations encompassing the fundamentals, optimization strategies of separation methods, and applications in metabolomics are found in the literature.[56,58,59]

LC can be coupled to MS using different ionization sources, in which electrospray (ESI) in positive and negative modes being the most applied. ESI has been shown to be very efficient in the ionization of large biomolecules and small metabolites, such as sugars, sterols, steroids, amino acids, phospholipids, fatty acids, among others. LC-MS combines the high robustness, resolving power, and selectivity of LC with the high sensitivity of MS, and no need for chemical derivatization, allowing a wide range of metabolome coverage. The prior separation of chemical species reduces the complexity of the matrix, reducing ionization suppression problems, which are often encountered in direct MS analysis methods.[60] Advances in MS

systems include the development of fast data acquisition rate, mass accuracy, and sensitivity for a wide range of *m/z*.[61] Different mass analyzers provide valuable information to assist in one of the most challenging steps of the metabolomics workflow: accurate metabolite annotation. Triple quadrupole (QqQ), quadrupole time of fight (QToF), and Orbitrap (OT) instruments are used. These analyzers allow the performance of tandem-MS (MS^n) analyses, which enable the accurate structural elucidation of metabolites based on the search for fragments generated in spectral libraries or using authentic analytical standards.[56]

The possibility of applying different analysis strategies is especially interesting regarding microbial metabolomics due to the high complexity of metabolites from interactions in microbiomes.[50] A method based on HILIC-MS using an OT-MS was used to understand the mechanisms involved in combining different treatments against *Pseudomonas aeruginosa*.[38] RPLC-MS and HILIC-MS were used to understand the influence of probiotics on the growth and biotransformation of four *Lactobacillus* strains, demonstrating different profiles of cellular adaptation to the environment.[62] Another interesting work developed a new approach using two zwitterionic columns in HILIC-MS analysis to quantify microbial boundary fluxes. This new strategy, focusing on the *Staphylococcus aureus* exometabolome, determined of almost 400 metabolites in less than 5 min of analysis. This method demonstrated high precision for more than 1000 injections, allowing the identification of microbial species in different clinical conditions.[63]

CE-MS

Compared to the chromatographic separation techniques, capillary electrophoresis mass spectrometry (CE-MS) has been the least applied analytical technique in metabolomics investigations. The separation mechanism involved in CE differs slightly from LC and GC and is based on the differentiated migration of ionic or ionizable solutes under an electric field.[64] Different separation modes are used, such as capillary zone electrophoresis (CZE) for small and large ionic molecules; micellar electrokinetic chromatography (MEKC) for neutral species; capillary isotachophoresis (CITP) for small ionic molecules; capillary gel electrophoresis (CGE) for large biomolecules and polymers; among others.[65] Due to its versatility, this orthogonal technique is suitable for the determination of the polar and ionic portion of the metabolome, with the advantage of not requiring derivatization. Furthermore, CE combines high resolving power and separation efficiency, achieved by the planar electroosmotic flow (EOF) profile formed inside the capillary and the small volumes of sample injected. It was a very interesting technique for application in biological studies, especially with sample volume restriction.[48,66] Sample handling can be minimal, including cell lysis, dilution, and protein removal.[48] Compared to LC, CE has the advantage of not requiring large volumes of organic solvents for solute elution and shorter analysis times. On the other hand, its presents lower sensitivity since it works with a small volume of injected sample (nL) and poor migration time reproducibility due to fluctuations of EOF.[64]

The CE-MS coupling, generally performed via electrospray (ESI), emerged in the late 1980s and proved advantageous for its sensitivity and selectivity, with lower limits of detection (LODs) when compared to the frequently used UV-Vis methods. However, CE-MS coupling is not as simple as LC-MS and GC-MS due to the high electric field and low eluent flow rates (low EOF), which maintain unstable electrospray formation.[66] Two CE-MS interface configurations are found: coaxial sheath liquid and sheathless interface. The first has a system configured for continuous pumping of a sheath liquid (SHL), composed of mixtures of organic solvents (acetonitrile, methanol, or isopropanol) with acidified aqueous solutions (formic or acetic acid) and volatile additives (ammonium salts). The SHL is mixed with the CE eluent in order to improve the ionization of the analytes, promoting the stability of the spray formed in the ESI. Optimizations of the composition of the background (BGE) and the SHL are critical and must be performed together to obtain the maximum ionization of the analytes for both positive and negative ionization modes.[67]

Despite being widely applied, the SHL interface considerably reduces the detection sensitivity by diluting the eluent. In order to circumvent such compromise, a technology based on a sheathless interface was developed by Moini.[68] In this configuration, the coated (metal or polymeric) porous tip of the capillary improves spray formation and mass transfer by directly inserting eluent from the CE into the MS system by

applying a voltage at the capillary outlet. This configuration was applied in metabolomics for the first time in 2012 to characterize the metabolic profile of human urine[69] and has since been used in cellular,[70] tissue samples,[71] and body fluids (urine, plasma, and cerebrospinal fluid),[72] among other studies. Configuration changes and improvements in the prototypes, such as the incorporation of a nanocapillary electrophoresis-MS (nanoCESI), allowed pre-concentrations and increased system sensitivity.[73] However, some limitations must still be considered, such as short capillary lifetime, variation in EOF, and low repeatability and robustness.[65]

The development of new CE-MS interfaces and technologies for volume-restricted investigations have increased their use in metabolomics in the last ten years (about 30-40%, according to PubMed search using *"metabolomics"* and *"CE-MS"* keywords). Despite the challenges and limitations, the conventional interface (co-axial sheath liquid) is still been the most applied. Compared with other analytical platforms, CE-MS is also considered a limited technique, attributed to the difficulty of establishing standard protocols, low reproducibility, and the lack of experts in the field.[74] Regarding microbial metabolomics, its application is even more incipient. A recent work evaluated the metabolic profile of the response of *Schefersomyces stipitis* to N-acetyl-d-glucosamine (GlcNAc), an amino sugar used as an abundant renewable carbon source. Intracellular analysis by CE-ToF-MS allowed a better understanding of fungal metabolism, demonstrated by the increase in nitrogen-containing metabolites known for biological and pharmacological properties.[75] Another interesting investigation using CE-MS and LC-MS was performed by Yamamoto and collaborators,[76] showing differences between small and large intestinal metabolic profiles between specific pathogen-free and germ-free mice. The findings suggested that differences in the functions of each part of the intestinal tract are associated with the colonization of different microbial species, resulting in specific metabolites related to inflammatory processes. Understanding interactions and associations with inflammation can help improve health promotion.

APPLICATIONS OF CLINICAL MICROBIAL METABOLOMICS

Microbial metabolomics has been a prominent area within the omics sciences, given the importance of MO for understanding cellular mechanisms. As previously mentioned, research articles in microbial metabolomics have increased linearly in the last years (Figure 1). Some revisions including methods and new findings have been published recently.[16,25,77] Among these, some works involving the discovery of new drugs, resistance to antibiotics, intestinal microbial metabolism comprehension, metabolic engineering, and biotechnological processes (such as fermentation) have been the focus of researchers in the field.[17] Clinical applications involving the understanding of pathologies associated with MO and the development of diagnostic tools and new treatments have also been of interest. An untargeted investigation by GC-MS evaluated the metabolic alterations provoked by the biofilm community of *Candida albicans* and *Klebsiella pneumoniae*. The analysis of the endometabolic profile of individual and dual biofilms showed marked differences (Figure 3), in which 40 metabolites with significant alterations were identified. Such compounds were associated with the maturation of biofilms. They could be an important starting point for developing strategies to combat infections caused by these opportunistic MOs, promoting quality of life and reducing mortality in hospitalized patients.[78] The metabolic response of *Escherichia coli* was evaluated in a multi-omics, polar metabolomics, and lipidomics study to understand adaptations to acid stress and amino acid supplementation in environments such as the stomach and intestine. *E. coli* is an opportunistic pathogen responsible for severe urinary tract and intestinal infections. The results indicated a coordinated correlation between amino acid-dependent mechanisms for acid resistance and lipidomics modulations, emphasizing lipid synthesis routes and metabolite transport changes. According to the authors, complementary studies need to be carried out to understand the mechanism and acid resistance to the gut environment. However, the initial findings help elucidate this MO's adaptation to develop new treatments for pathogenic organisms.[79] Table II presents a compilation of selected applications, described throughout this review, including the objectives and brief descriptions of the analytical methods used.

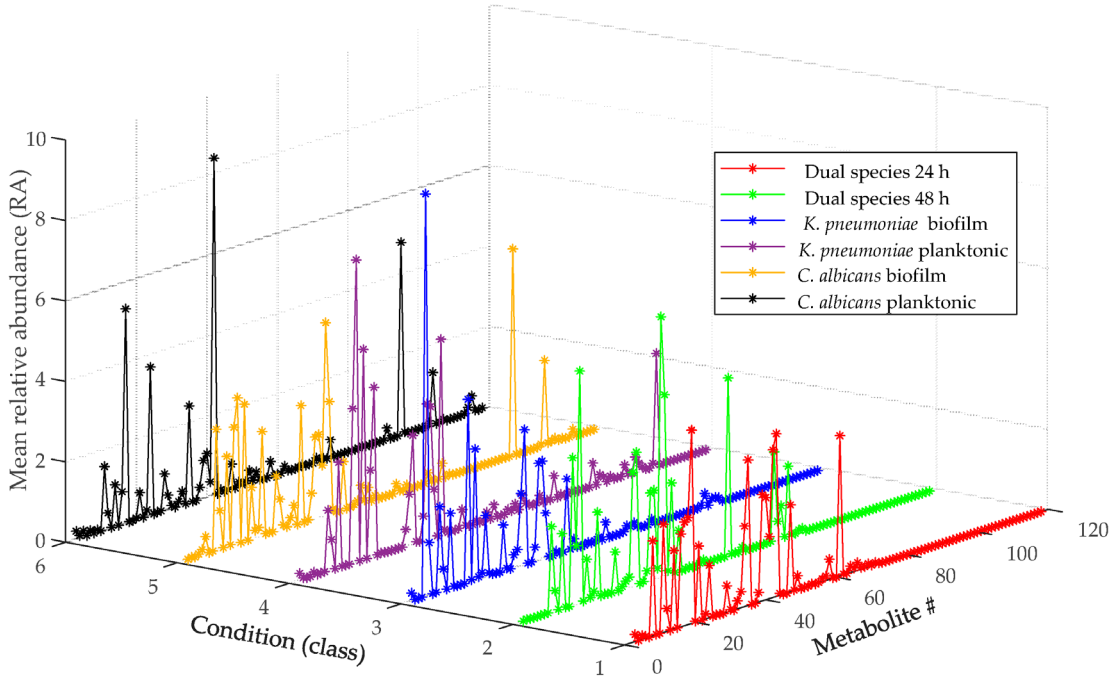

Figure 3. Extracellular metabolic profiling analyzed by GC-MS of the six conditions studied in the biofilms of *Candida albicans* and *Klebsiella pneumoniae*.

[Reprinted under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/) from reference No. 78: Galdiero, E.; Salvatore, M. M.; Maione, A.; de Alteriis, E.; Andolfi, A.; Salvatore, F.; Guida, M. GC-MS-Based Metabolomics Study of Single- and Dual-Species Biofilms of *Candida albicans* and *Klebsiella pneumonia*. *Int. J. Mol. Sci.* **2021**, *22* (7), 3496. http://dx.doi.org/10.3390/ijms22073496.]

Table II. Selected applications and analytical methods used in mass spectrometry-based microbial metabolomics

Microorganism (Sample type)	Aim	Quenching	Extraction Methods	Analytical Platform (Ionization modes)	Ref.
Aphanizomenon flos-aquae and *Microcystis aeruginosa* (cell pellet)	To understand the effect of *Microcystis aeruginosa* on *Aphanizomenon flos-aquae* growth.	Liquid N_2	Cellular extraction by homogenization at 35 Hz (4 min) with MeOH/H_2O (3:1, v/v), followed by sonication (5 min) in an ice-water bath (procedure repeated twice).	RPLC-MS/MS (positive and negative)	12
Azospirillum brasilense (cell pellet)	To evaluate the effect of growth of wild-type and mutant *Azospirillum brasilense* under low and high nitrogen.	Liquid N_2	Cellular extraction with MeOH 80% and sonication for 60 min. Dried extracts were derivatized by methoxyamination (50 ºC until residue resuspension) and silylation (60 min at 50 ºC).	GC-MS	13
Candida albicans, Candida glabrata, and *Candida tropicalis* (culture medium)	To differentiate *Candida* spp. by exometabolomics strategy.	**	SPME extraction using a fused silica fiber (divinylbenzene/carboxen/ polydimethylsiloxane). The headspace extraction occurred under agitation (350 rpm) at 50 °C for 30 min.	GC-MS	14
Bacillus licheniformis (cell pellet)	Optimization of sample preparation of *Bacillus licheniformis* for GC-MS untargeted metabolomics.	60% MeOH 0.9% NH_4HCO_3	Cell disruption with bead-milling and MeOH 70% solution (at -40 °C). Dried extracts were derivatized by methoxyamination (30 ºC for 90 min) and silylation (37 ºC for 120 min).	GC-MS	31
Corynebacterium glutamicum (cell pellet)	Optimization of sample preparation of *Corynebacterium glutamicum* for LC-MS untargeted metabolomics.	40% MeOH (-20 ºC)	Extraction with EtOH/H_2O (3:1, v/v) at 100 °C, incubation in boiling-water bath (15 min) and disruption in a grinder system (5 M/s, 30 s, 5 cycles) (2x). Dried extracts resuspended in ACN/H_2O (1:1, v/v, with 0.1% F.A.).	HILIC-MS (negative)	32
Candida albicans (cell pellet)	To study the mechanism of action of basil essential oil in *Candida albicans.*	Liquid N_2	Cell homogenization with 50% (v/v) cold MeOH and incubated at -20 ºC (30 min) and re-extraction with MeOH/$CHCl_3$ (3:1, v/v). Combined organic phases were dried and derivatized by methoxyamination and silylation, both performed at 37 ºC for 120 min.	GC-MS	33
Escherichia coli (cell pellet)	To evaluate the mechanism of colistin resistance mcr-1-mediated in *Escherichia coli.*	Liquid N_2	Pure MeOH (at -20 ºC) vortexed for 1 min. Cell lyse by freeze-thaw cycles in liquid N_2 (3x). Re-extraction with same conditions. Combined supernatants were dried and resuspended in ACN/H_2O (50/50 v/v, with 0.1% F.A.).	RPLC-MS (positive and negative)	34

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Table II. Selected applications and analytical methods used in mass spectrometry-based microbial metabolomics (continuation)

Microorganism (Sample type)	Aim	Quenching	Extraction Methods	Analytical Platform (Ionization modes)	Ref.
Saccharomyces cerevisiae (cell pellet)	Optimization of sample preparation of *Saccharomyces cerevisiae* for GC-MS untargeted metabolomics.	Fast filtration	Cells were extracted with ACN/H_2O (1:1, v/v) and glass beads by vortexing for 3 min. Dried extracts were derivatized by methoxyamination (30 ºC for 90 min and 200 rpm) and silylation (37 ºC for 30 min and 200 rpm).	GC-MS	35
Pseudomonas fluorescens, Streptomyces coelicolor, and *Saccharomyces cerevisiae* (cell pellet)	Optimization of quenching method for microbial metabolomics.	Glycerol/NaCl solution (13.5 g L^{-1}) (3:2, v/v)	Cell homogenization with 50% (v/v) of MeOH/H_2O (3:2, v/v, at -30 ºC) by vortexing (1 min), followed by freeze-thaw cycles (3x). The residual pellet was re-extracted with the same MeOH/H_2O solution by vortexing. Combined extracts were resuspended in NaCl (1 mol L^{-1}), MeOH, and pyridine, followed by derivatization with methyl chloroformate.	GC-MS	37
Pseudomonas aeruginosa (cell pellet)	To understand the mechanism of action of combined antibiotics (polymyxin and amikacin) against susceptible and resistant *Pseudomonas aeruginosa.*	ND	Freeze-thaw cycles (3x) in combination with $CHCl_3$/MeOH/H_2O (1:3:1, v/v/v) were used for intracellular extraction.	HILIC-MS (positive and negative)	38
Acinetobacter baumannii (cell pellet and culture medium)	To understand the mechanism of action of combined antibiotics (colistin and doripenem) against *Acinetobacter baumannii.*	Dry ice/EtOH bath	Freeze-thaw cycles (3x) in combination with $CHCl_3$/MeOH/H_2O (1:3:1, v/v/v, at -80 ºC) were used for intracellular extraction. Culture supernatant were mixed with $CHCl_3$/MeOH/H_2O (1:3:1, v/v/v) solution for extracellular extraction.	HILIC-MS (positive and negative)	39
Staphylococcus aureus (cell pellet)	To detect altered metabolites in *Staphylococcus aureus* using targeted metabolomics.	ND	Pure cold MeOH was vortexed with the pellets (~ 1 min). The mixture was maintained at -20 ºC (20 min). Supernatants were dried and resuspended in ACN/H_2O (1:1, v/v).	HILIC-MS (positive and negative)	41
Cobetia marina (cell pellet)	To understand metabolic changes in *Cobetia marina* under ozone stress.	Aqueous NaCl (0.85%, v/v) frosted into ice bulks, followed by storage at -80 ºC (3 min)	Cell disruption in a speed homogenization system (1 min) with 50% $CHCl_3$/ 50% MeOH/H_2O (21:79, v/v). Procedure repeated twice. Upper phases were combined and dried, followed by derivatization by methoxyamination and silylation, both performed at 60 ºC (60 min).	GC-MS	51

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Table II. Selected applications and analytical methods used in mass spectrometry-based microbial metabolomics (continuation)

Microorganism (Sample type)	Aim	Quenching	Extraction Methods	Analytical Platform (Ionization modes)	Ref.
Mycobacterium spp. (cell pellet)	To investigate potential volatile organic biomarkers that differentiate mycobacteria.	Ice bath	Samples were incubated for 15 min under 250 rpm and exposed to a SPME fiber (polydimethylsiloxane/carboxen/ divinylbenzene) for 20 min at 37 ºC.	GC-MS	53
Aspergillus carbonarius and *Aspergillus ochraceus* (cell suspension)	To understand the alterations in volatile organic compounds by the mycotoxin (ochratoxin A) contamination in *Aspergillus*.	ND	Samples were equilibrated for 30 min at 50ºC under agitation and exposed to a SPME fiber (polydimethylsiloxane) for 30 min.	GC-MS	54
Staphylococcus aureus (cell pellet)	To optimize an HILIC-MS method for quantification of microbial boundary fluxes.	Cold MeOH and -80 ºC storage	Supernatants from quenched samples were diluted with 50% MeOH at 1:10 (v/v).	HILIC-MS (positive and negative)	63
Scheffersomyces stipites (cell pellet)	To understand metabolic effects of *N*-acetyl-d-glucosamine as carbon and nitrogen source in *Schefersomyces stipites.*	MeOH (-40 ºC)	Boiling EtOH 75% (v/v) was added to the pellets and extraction was performed in a water bath at 95 °C. Samples were dried and resuspended in ultrapure H_2O.	CE-MS (positive and negative)	75
Not specified (mice intestinal tissue)	To investigate differences into the metabolome of intestinal luminal and correlate with commensal microbiota in mice.	Storage at -80 °C	Pure MeOH and zirconia beads were vortexed with the sample tissues, containing PBS (0.957 mmol L^{-1}). After protein precipitation, supernatants were concentrated and dissolved in H_2O.	CE-MS (positive and negative), and LC-MS (negative)	76
Candida albicans and *Klebsiella pneumoniae* (culture medium)	To investigate the metabolic changes in single- and dual-species biofilm development.	**	Supernatants were dried and derivatized by silylation, in which rotated samples were exposed to a stream of air (50-120 ºC).	GC-MS	78
Escherichia coli (cell pellet and culture medium)	To characterize the *Escherichia coli* adaptations to acid stress.	ND	Cold MeOH were vortexed with the pellets and culture medium supernatants. Samples were maintained at -20 ºC (20 min) to precipitation and further analysis of supernatants.	LC-MS (positive and negative)	79

ACN, acetonitrile; EtOH, ethanol; F.A., formic acid; MeOH, methanol; ND, not described.
**Extracellular metabolomics does not require quenching step.

CONCLUSIONS

Given the increased interest in understanding the metabolic relationships and interactions between microorganisms and hosts, metabolomics appears to be an appropriate study tool. The great complexity of the metabolome of microorganisms makes such studies challenging, especially in the analytical aspects of sample preparation and data acquisition. Sample harvesting and metabolic quenching must be carefully planned according to the studied organism. Cellular metabolism disruption without metabolite leakage or degradation during quenching requires optimization. The analysis of intracellular metabolome also demonstrates a lack of standard methods. Systematized studies of extractor solvent composition and cell membrane rupture methods are encouraged. The variable chemical diversity in microorganisms means that more than one analytical technique is required for broad metabolome coverage. The use of separation techniques coupled with mass spectrometry has provided excellent results, given the high versatility and separation power, combined with the detectability and sensitivity of the MS. LC-MS has undoubtedly been the most applied platform, using different elution modes. GC-MS arises as the gold standard for elucidation of the volatile metabolome. Despite requiring an additional step of derivatization in sample preparation, methods based on microextraction are used efficiently. Finally, despite being little explored, CE-MS requires little sample volume and proves useful for highly polar and ionic metabolites, complementing the information on the metabolic profile obtained by the other analytical platforms.

Thus, with the chemical complexity of the studied organisms and recent technological advances, the analytical chemistry field occupies an important niche within metabolomics, including investigations in microbial metabolomics, in which new optimizations and methods developments are often needed.

Conflicts of interest

The authors declare no financial conflicts of interest.

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