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Rustam Aminov

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REVIEW

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Metabolomics in antimicrobial drug discovery

Rustam Aminov

School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, UK

ABSTRACT

Introduction: In light of the ever-escalating problem of antimicrobial resistance, there is an urgent need for the development of new antimicrobials. In this review, the role of metabolomics in antimicrobial drug discovery and development is summarized and discussed. For this, ScienceDirect, PubMed, Web of Science and Google Scholar databases were searched with the article's keywords and their combinations to retrieve the most relevant and up-to-date information.

Areas covered: The areas covered include the metabolomic concepts and techniques and bioinformatic tools used in metabolomics as well as recent developments in these areas. Also, examples of the use of metabolomics tools in several areas of antimicrobial drug discovery are given.

Expert opinion: Metabolomics, with the corresponding bioinformatic support and combination with other omics technologies, represents an integral and essential part of antimicrobial drug discovery and development. Metabolomics contributes to the mechanism-based approach in antimicrobial drug discovery, reveals the mechanisms of action of antimicrobials and non-antimicrobial compounds, identifies new targets, and opens new ways to manage and control bacterial infections.

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Metabolomics; antimicrobials; drug discovery; liquid chromatography; gas chromatography; mass spectrometry; nuclear magnetic resonance; metabolomics software; metabolomics database; ecosystem metabolomics

1. Introduction

The term 'metabolome' was first introduced in 1998 to explicate that microbial cells have to vary metabolite concentrations in order to maintain constant metabolic fluxes [1]. The aim of metabolomic analysis is to determine the complete profile of metabolites present in a biological system, with a time and spatially defined metabolite abundance. The biological systems could be cells, biofluids, tissues, organisms, and even ecosystems, while metabolites are usually small molecules with a molecular weight <1500 Da [2,3]. Unlike other omics approaches such as genomics, transcriptomics, and proteomics, which are generally gene-centric and thus can be reasonably well integrated within a genomic context, the metabolome represents the complex and nonlinear products, which results from differential regulation at the genomic, transcriptomic, and proteomic levels and which, in addition, are confounded by epigenetic and post-translational modifiers, redundant connections between metabolites, and a variety of environmental factors. Thus, the products of biochemical reactions in the form of metabolites are much more complex and also could be below the limits of our current analytical techniques. In this respect, the development of integrated metabolism-centric databases and software is a crucial prerequisite for the efficient use of metabolomics approach by researchers [4].

In the context of antimicrobial drug discovery, the metabolomics approach can be applied in several fields. The well-known classical approach is the analysis of secondary metabolites produced, largely in the secreted form, by soil bacteria, particularly Actinomycetes. Among the collection of secondary metabolites, which are produced by bacteria, fungi, plants, and sponges, some may display antimicrobial activities, and, with the use of metabolomics, these compounds can be identified, purified, and characterized. Second, the effect of chemical compounds can be tested using a model pathogen, to investigate how the compounds may affect the metabolome/metabolic fluxes of a microorganism tested. This allows identifying targets and reveals mode of action of the compounds tested. This approach is especially useful at the early drug development stage to characterize lead compounds that may have limited and non-lethal activity toward a pathogen. The third aspect is the effect of an antimicrobial drug on the host metabolome. These interactions may range from benign side effects to acute toxicity. With the decreasing arsenal of antimicrobial drugs due to the rising antimicrobial resistance, it becomes necessary to recruit the drugs, which in the past have been considered too toxic for the routine use such as colistin. Thus, the area of toxicometabolomics [5] becomes more important in the era of scarce antimicrobial drug supply. Metabolic signatures due to antimicrobial drug toxicity allow identification of metabolic pathways affected and correspondingly evaluate the risks imposed to particular patient cohorts.

For the purpose of this review article, the next databases were searched: ScienceDirect, PubMed, Web of Science, and Google Scholar. The search terms were metabolomics, antimicrobials, drug discovery, liquid chromatography, gas chromatography, mass spectrometry, nuclear magnetic resonance, metabolomics software, metabolomics database, and ecosystem metabolomics. The search terms were also compounded

CONTACT Rustam Aminov rustam.aminov@abdn.ac.uk School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

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Article highlights

- Recent developments in metabolomics techniques are characterized by high-throughput, smaller sample size, higher sensitivity, broader coverage of metabolites, faster analysis, decreased cost, miniaturization, and automation.
- In bioinformatics support of metabolomics, the main trends are the development of freely available open-source software and public databases, standardization of data reports for data sharing and analysis, and the development of software platforms providing the complete workflow for large amounts of data.
- In antimicrobial drug discovery and development, the majority of antimicrobials in the late clinical stage are still from the existing classes of antimicrobials. Thus, there is an urgent need for novel antimicrobial classes to curb the growing resistance problem.
- Metabolomics-enabled and mechanism-based approach in antimicrobial drug discovery allows to broaden the sampling base and assess the ecological niches that previously, with the use of activity-based methods, were difficult to analyse. Besides, high-throughput automated metabolomic approach provides fast dereplication of antimicrobials from natural and synthetic sources, thus expediting antimicrobial drug discovery process and potentially identifying compounds with novel MoAs.
- Mechanism-based metabolomics approach allowed to identify non-antimicrobial compounds that affect bacterial metabolism and thus could be a basis for combination therapy. The link between the bacterial metabolome and virulence could be exploited to modulate virulence via metabolome and thus limit damage to the host.
- Metabolomics approach also plays an important role in identification of antimicrobial mode of action, drug–target characterization, drug–drug interaction, and drug repurposing.
- In summary, metabolomics-aided and mechanism-based approach opens an entirely new era in antimicrobial drug discovery and development.

in different combinations to improve the search results for the most relevant and up-to-date information. Literature search was performed from April 1 to 30 April 2022.

2. Metabolomic principles and techniques

Metabolomic analyses are essentially consisted of sample preparation, separation, and detection. Once a sample is collected, all metabolic reactions should be extinguished in order to have a snapshot of all metabolites present at a given time and conditions. Samples can be preserved by flash freezing in liquid nitrogen, freezing on dry ice, or adding organic solvents. The latter could be considered as a part of the extraction step, which is aimed at collection of as many metabolites as possible, while trying to preserve their chemical structure. This is usually accomplished by solid-phase or liquid–liquid extraction, where the compounds of interest are placed in a liquid mixture, which allows their separation from other compounds, thus concentrating the sample and removing impurities that may interfere with further analyses [3].

In most cases, the metabolite profiles are extremely complex, and a separation step is necessary before the metabolite detection step. The two most widely used metabolite separation techniques are gas chromatography (GC) and liquid chromatography (LC). The most widely used detection techniques for metabolic analyses are mass spectrometry (MS) [6] and nuclear magnetic resonance (NMR, with the most important nuclei ^{1}H, ^{13}C, ^{15}N, and ^{31}P).

The main combined analytical tools in metabolomics include liquid chromatography coupled to mass spectrometry (LC-MS) and gas chromatography coupled to mass spectrometry (GC-MS). Less commonly used metabolomic technologies are mainly associated with MS techniques and include direct injection, capillary electrophoresis coupled to mass spectrometry (CE-MS), ultra-high performance liquid chromatography coupled to mass spectrometry (UPLC-MS), high-performance liquid chromatography-electrospray ionization coupled to mass spectrometry (HPLC-ESI-MS), high-performance liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), ultra-high-performance liquid tandem chromatography quadrupole time of flight mass spectrometry (UHPLC-QTOF-MS), inductively coupled plasma-mass spectrometry (ICP-MS), and others.

Currently, the number of compounds resolved by NMR lags behind MS substantially, mainly because of lower sensitivity, although it may offer certain advantages in reproducibility, wide dynamic range, determining the structure of unknown compounds, and sample preservation [7]. Besides, although to a much lesser extent, Fourier-transform infrared or Raman scattering spectroscopy are used in metabolomic research. In a survey of metabolome researchers (with some of them using multiple technologies across their studies), a considerable majority of respondents utilized LC-MS (83%), followed by GC-MS (30%), NMR (26%), DIMS (16%), imaging mass spectrometry (6%), MALDI-MS (4%), and CE-MS (3%) [8]. Thus, the prevailing majority of metabolomic research is currently performed with the use of the three most popular technologies, LC-MS, GC-MS, and NMR.

Compared to GC-MS and NMR, LC-MS is the most widely used technique due to the wealth of information it is capable of providing as well as due to its sensitivity, flexibility, and versatility. Developments in this technology include the use of smaller particle-size packed columns, decreasing mobile-phase viscosity, and optimization of column technology to improve the efficiency of separation by reducing eddy and longitudinal diffusion and improving mass-transfer resistance [3]. The use of multi-dimensional separations such as two-dimensional LC may also improve separation efficiencies and extend the coverage of metabolites. Other developments in the field include integration of microfluidic and digital devices with MS, which, due to miniaturization, allows incorporation of different components performing sample preparation, separation, and introduction steps. Commercially available devices include IonKey (Waters) and HPLC-Chip (Agilent), chipLC-ESI devices, and the ZipChip (908 Devices) chipCE-ESI device [3]. Integration of digital microfluidics platforms with MS offers even further miniaturization, smaller sample size, faster analysis, high-throughput, and automation.

Compared to LC-MS, the use of GC-MS in metabolomics is relatively modest, mainly due to the restricted range of metabolites it is capable of analyzing. This range includes volatile and thermally stable compounds, with some more metabolites that can be rendered volatile via chemical derivatization [9]. Despite this limitation, GC-MS retains its place as one of the oldest and well-established techniques, notable for its

separation capacity, sensitivity, and selectivity. It is highly reproducible and benefits from the availability of extensive libraries and databases for metabolite identification. Improvements of the separation capability of GC such as the use of 2D-GC (2-Dimensional GC), which combines two orthogonal columns together at the separation step, may significantly increase the separation efficiency and therefore contribute to the detection of a larger number of metabolites compared to 1-Dimensional separation (1D-GC) [10].

The most frequently used NMR technique in metabolomics is 1D-NMR (1-Dimensional NMR). 2-Dimensional NMR (2D-NMR), which is represented by two frequency axes, is used to resolve molecular structures that are difficult to determine with 1D-NMR spectroscopy. Certain advantages of NMR technique in metabolomics are due to the broad coverage of metabolites, absence of the metabolite separation stage, and its non-destructiveness so that NMR samples can be reused in other analyses. Besides, compared to MS, NMR is certainly more quantitative, with a signal intensity directly proportional to the concentration of metabolites [11]. Problematic, however, remains its low resolution due to signal overlap, especially in biofluids, and a relatively low sensitivity so that metabolites should be present at sufficiently high concentrations in order to be detected and quantified. Further development of NMR for metabolomics will require increased sensitivity such as with the use of microcoil NMR and hyperpolarization, improvement of spectral processing techniques [3] and further expansion of NMR spectral libraries.

Metabolomic approaches can be targeted or untargeted [12]. In targeted metabolomics, defined groups of known, chemically characterized and biochemically annotated metabolites are monitored, temporally or as a response to a system perturbation. With the use of internal standards, the concentration of metabolites can be evaluated, quantitatively or semi-quantitatively. Contemporary techniques such as LC-MRM/PRM-MS-based analytical strategies allow quantitative analyses of several hundred metabolites, with a wide linearity of the measurement range and high reproducibility [13]. In antimicrobial drug discovery research, this approach can be used to identify the mode of action of a potential drug lead or reveal potential side effects on the host. Thus, multiple reaction monitoring (MRM), parallel reaction monitoring (PRM), and selected reaction monitoring (SRM) could be performed to identify antimicrobial drug targets, both in a pathogen and a host, with the aim of maximizing the drug effect on the former and minimizing it on the latter.

In antimicrobial drug discovery, the use of targeted metabolomics apparently has limitations since the approach deals with the fixed pool of known metabolites, which restricts metabolite coverage and thus may miss important metabolites. The use of targeted metabolomics can be supplemented with untargeted approach to address concerns about potentially missing metabolites. Untargeted metabolomics is aimed at a complete analysis of all measurable metabolites, including unknown ones, and this approach is certainly the method of choice in the discovery of novel antimicrobial compounds.

There are many challenges intrinsic to the untargeted approach though, with high complexity and voluminous amounts of raw data generated, potential biases associated with high-abundance metabolites and analytical range of the platforms used, ambiguities with identifying and characterizing unknown metabolites, and excessive reliance on the comprehensiveness of metabolite databases used for analysis. With addressing these challenges and with the rapid development of bioinformatic tools and corresponding open access spectral and chemical databases [14], the implementation of untargeted metabolomics approach becomes more widely accepted and used in a variety of metabolic applications [15]. The general trend in metabolomics is a further development of technologies that provide faster and more exhaustive analyses, use smaller samples, and decrease the cost of analysis [3]. A recent overview of miniaturized metabolomics for biomass-restricted samples, including single-cell metabolomics, has explored the development of corresponding techniques, including sample preparation procedures, separation techniques, and MS analyzers [16].

3. Metabolomics: bioinformatic tools

Bioinformatic analyses used in metabolic studies are primarily aimed at correctly identifying metabolic products, providing quantitative assessment of metabolites, contributing to metabolic reconstruction, revealing metabolic features, and identifying metabolic effects of environmental variables. Data from metabolic analyses come in a broad range of MS and NMR spectra. Hardware for metabolomics usually comes with the software bundled, which can be adequate for routine analyses to perform data processing, peak annotation, metabolite identification, and statistical and pathway analyses. For more advanced metabolomics applications, however, these capabilities may not be sufficient. Moreover, since the software provided and data formats are proprietary, there is an incompatibility problem among different processes and different software versions. Also, there could be limitations in spectral database access, which is especially problematic for untargeted metabolomic analyses. Thus, there is an increasing number of free to use open-source software tools that can perform one or more of these tasks. A comprehensive list of these bioinformatic tools for metabolomics was compiled by Spicer and others [17].

The prevalence of utilization of open-source and free-to-use software tools vis. commercial software bundled with instruments by the metabolomics community has been recently surveyed by Weber and others [8]. Among LC-MS users, 84% used open-source software, while 65% – commercial software bundled with the instrument (some users used both, hence the number is higher than 100%). Among the open-source software users, about 70% used XCMS [18, https://xcmsonline.scripps.edu, accessed on 13 July 2022]. XCMS Online processes LC-MS data for untargeted metabolomics profiling and provides a complete metabolomics workflow including feature detection, retention time correction, alignment, annotation, and statistical analysis. The second in popularity (26%) were mzMine and its updates [19,20, https://mzmine.github.io/, accessed on 13 July 2022]. Among GC-MS users, commercial software bundled with the instrument was used by 76%, with the open-source users at 67% [8]. Commercial software users mostly employed Agilent

ChemStation (45%), whilst the preferred open-source software was AMDIS (http://amdis.net, accessed on 13 July 2022) at 50% and the previously mentioned XCMS (40%). In NMR spectroscopy, the use of commercial software was much more prevalent [8]: 78% of respondents used commercial software bundled with the instrument and 50% – the third-party commercial software. A leading commercial software utilized was Bruker's TopSpin (56%), with a much lower number of non-commercial software users, who employed NMRlab/MetaboLab [21, https://github.com/RASpicer/MetabolomicsTools/blob/master/tools/MetaboLab.md, accessed on 13 July 2022] and rNMR [22, http://rnmr.nmrfam.wisc.edu, accessed on 16 July 2022], both at 17%. Thus, the open-source XCMS is a predominant software for processing of MS data, while NMR data are mainly processed with the use of commercial software.

Among the recent developments of vendor-independent open-source software tools for targeted MS is Skyline [23, https://panoramaweb.org/SkylineForSmallMolecules.url, accessed on 13 July 2022]. This software is able to support bioinformatic analysis from raw data produced by mass spectrometers of several vendors such as Sciex, Waters, Agilent, Bruker, Shimadzu, and Thermo. The authors indicated that Skyline has become a versatile and powerful open-source software solution for quantitative MS data workflows, which can support both proteomics and metabolomics data processing in a vendor-neutral environment, improve transparency, and facilitate data and methods exchange [23].

Metabolite identification and annotation remains the most time-consuming bottleneck step in metabolomics [24]. Minimum reporting standards related to metabolite identification were defined and updated several times [25–27]. Requirements to level 1 identification by Metabolomics Standards Initiative (MSI) include at least two independent orthogonal data that match an authentic compound measured under identical experimental conditions in the same laboratory. Many metabolites are not available commercially and although many software packages assert metabolite identification, they can only provide putative identification at level 2 [17]. Putative identification is still useful and about 80% of MS users conducted metabolite annotation using full scan MS1 data [8]. For annotation, 70% used CAMERA [28, https://bioconductor.org/packages/release/bioc/html/CAMERA.html, accessed on 13 July 2022], which is freely available from the Bioconductor repository for Windows, Mac OS, and Linux operating systems. Data produced with Multi Stage Mass Spectrometry (MS^n) were annotated with a broader range of software [8], including Metlin [https://metlin.scripps.edu, https://massconsortium.com; both sites accessed on 13 July 2022], MetFrag [https://msbi.ipb-halle.de/MetFrag, accessed on 13 July 2022], XCMS, and RMassBank [https://bioconductor.org/packages/release/bioc/html/RMassBank.html, accessed on 13 July 2022] within Bioconductor [https://bioconductor.org/, accessed on 13 July 2022]. AMDIS and the NIST Mass Spectrometry Data Center [https://chemdata.nist.gov, accessed on 13 July 2022] were the preferred solutions for annotation of GC-MS data [8]. Annotation of NMR data largely relied on the use of commercial software, these are NMR Chenomx's NMR suite [https://www.chenomx.com, accessed on 13 July 2022] and AMIX from Bruker [https://www.bruker.com/en/products-and-solutions/mr/nmr-software/amix.html, accessed on 13 July 2022], each with 39% of users [8]. Open-source software programs for this purpose include the Birmingham Metabolite Library [29, http://www.bml-nmr.org, accessed on 13 July 2022] and other data mining tools such as BATMAN [30, http://batman.r-forge.r-project.org/, accessed on 13 July 2022] and rNMR, with 22%, 17%, and 9% users, respectively.

There is also a family of databases, called KNApSAcK, which describes the metabolites produced by distinct biological species [31, http://kanaya.naist.jp/KNApSAcK_Family, accessed on 13 July 2022]. These databases cover mostly plant species but not exclusively, many bacteria, fungi, and other species are also included. These databases can be searched using molecular mass, molecular formula, metabolite name, or mass spectra in several ionization modes. These databases could be especially useful for those interested in metabolites of medicinal/edible plants that are used in traditional medicines.

Given the extraordinary diversity of instrumentation and software used, with wide experimental variables, databases for metabolomic data currently do not have standardized reporting formats. These standards are in the process of development, with the inclusion of various components such as the spectral and chromatographic data, the chemical structure associated, biological roles, locations, and concentrations, as well as metadata describing assays and the study as a whole. One of the metabolics repositories for a number of metabolomics journals is MetaboLights [https://www.ebi.ac.uk/metabolights, accessed on 13 July 2022] within EMBL. Other databases and repositories are MetabolomeExpress for GC-MS data [https://www.metabolome-express.org, accessed on 30 April 2022], Metabolomics Workbench [https://www.metabolomicsworkbench.org, accessed on 13 July 2022], Global Natural Product Social Molecular Networking (GNPS) [https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp, accessed on 13 July 2022], and NMR-based Human Metabolome Database [https://hmdb.ca, accessed on 13 July 2022].

As can be seen from this brief overview of bioinformatics supporting the metabolomics research, the field needs standardized data reporting for data sharing and analysis. Currently, one of the major problems in the area is incompatibility between different sets of software, from raw data to reporting formats. The development of automated and open-source and freely available software platforms that are capable of providing the complete workflow for large amounts of data, from pre-processing of raw data to the putative identification of metabolites and metabolic pathways, is a priority. It is argued that open data and data mining is an important step forward to maximize the potential of natural products research [32]. Some efforts toward this goal such as the development of platforms for analyzing, sharing, publishing, and referencing metabolomics data can be already seen [33–36].

Finally, there is a strong need for closer integration of metabolomics with other omics data to generate a better understanding of biological processes taking place within the genome-transcriptome-proteome-metabolome continuum in response to the environmental and other cues. A general overview of computational tools for performing large-scale

omics datasets integration was made by Fondi and Liò [37]. Pedersen and others [38] proposed a computing protocol for integration of multi-omics data that included dimensionality reduction technology: combination of data normalization approaches, binning of co-abundant genes and metabolites, and integration of prior biological knowledge. Some other functional implementations of the multi-omics approach include the Omics Discovery Index (OmicsDI), which integrates different omics data collections including genomics, proteomics, transcriptomics, and metabolomics [https://www.omicsdi.org, accessed on 13 July 2022]. Currently, OmicsDI integrates 23 databases, with each containing large datasets, and it is a part of the ELIXIR infrastructure, which is a European initiative to discover, access, integrate, and analyze biological data [https://elixir-europe.org, accessed on 13 July 2022]. Analysis of multi-omics data can also be performed with OmicsAnalyst, which allows visual analytics of submitted data [39, https://www.omicsanalyst.ca, accessed on 13 July 2022]. This platform also includes built-in annotations for transcriptomics, proteomics, metabolomics, and miRNA data for some common model organisms such as human and mouse. There is a number of other metabolomics and multi-omics data integration approaches, with the corresponding multi-omics tools and techniques supporting integrative analysis [40,41].

4. Metabolomics in antimicrobial drug discovery

4.1. Soil

In the beginning and during the golden age of the antibiotic era, the antimicrobial drug discovery programs were mainly focused on the investigation of soil bacteria, which produce numerous secondary metabolites with antimicrobial activity [42–44]. Technically, this approach was accomplished by monitoring the inhibition by a test strain, which is a suspected producer of antimicrobial(s), of an indicator strain/pathogen. This activity-based approach produced many classes of antimicrobials, but the last novel class, lincosamides, was discovered more than 50 years ago [43]. Presently, the prevailing majority of antimicrobial drugs in the late clinical development belong to the already existing antimicrobial classes, with only a few narrow spectrum compounds directed toward novel bacterial targets [45]. Strategies for discovery of new antimicrobials should be indisputably extended beyond the well-known soil Actinomycetes, and other ecological niches and taxonomic groups have to be explored. At the same time, we have to admit that the full potential of soil microbiota could still be under-explored due to the reliance on cultivable microbiota and on traditional activity-based screening methods, which still dominate the strategies of antimicrobial drug discovery. The use of advanced cultivation techniques such as iChip, which emulates environmental conditions, may help to improve the recovery of difficult-to-cultivate bacteria, and increase the throughput [46]. Also, metagenomic approach could help to recover and express DNA from uncultivated microbiota, which may contain gene clusters encoding for new antimicrobials [47]. Heterologous expression therefore may help to reveal antimicrobial potentials of silent biosynthetic gene clusters.

The application of untargeted metabolomic approach to soil may help to explore a broader diversity of soil organic matter composition, including secondary metabolites with antimicrobial activities, than it is possible with the activity-based pure culture approach [48]. The corresponding genetic constituents behind the production of these metabolites could then be isolated via metagenomic approach. Thus, the metabolomics tools could be complementary to the classical screening methods and facilitate prioritization of soil isolates, which, based on metabolome profiling and dereplication results, may produce novel antimicrobial compounds [49,50].

4.2. Commensals

Another ecological niche for exploration is the microbiome of mammals and humans, which has recently been recognized as a potential source of natural products, including secondary metabolites with therapeutic potential [51,52]. For instance, the isolation and characterization of indole propionic acid, which is produced by the human gut microbiota, and which is active against a broad spectrum of mycobacteria, including drug-resistant *Mycobacterium tuberculosis* and nontuberculous mycobacteria, suggested that this ecological compartment could be a source of novel antimicrobials [53]. Importantly, the antimicrobial activity of this metabolite is complemented by its anti-inflammatory and antioxidant activities, which further increases its value as a potential therapeutic agent. The gut microbiome may also be a rich source of metabolites with anthelmintic activity [54]. In this respect, systematic screening of metabolites produced by the human gut microbiota may lead to the discovery of other novel antimicrobials. Other compartments of human microbiota may also serve as a source of new antimicrobials. Nasal *Staphylococcus lugdunensis* strains, for example, produce lugdunin, a novel thiazolidine-containing cyclic peptide, which is active against major pathogens, efficient in animal models of infection, and causes no detectable resistance in *S. aureus* [55].

The value and potential of the human commensal microbiome is further enhanced by the fact that it is probably one of the best studied ecosystems in a multi-omics sense, with a multitude of databases covering various omics aspects such as the Human Microbiome Project [https://portal.hmpdacc.org, accessed on 14 July 2022], the Virtual Metabolic Human [www.vmh.life, accessed on 14 July 2022], the Unified Human Gastrointestinal Genome catalog [https://www.ebi.ac.uk/ena/browser/view/PRJEB33885, accessed on 14 July 2022], the relevant microbiome data within MGnify [https://www.ebi.ac.uk/metagenomics, accessed on 14 July 2022], GMrepo [https://gmrepo.humangut.info/home, accessed on 14 July 2022], and many others. Examination of metagenomic samples from the Human Microbiome Project, for example, allowed to identify a biosynthetic gene cluster, presumably encoded by *Lactobacillus gasseri*, which is responsible for the production of a thiopeptide antibiotic, lactocillin, with a potent antibacterial activity against a range of Gram-positive vaginal pathogens [56].

Presently, however, focused efforts toward analyses of metabolic pathways of commensal microbiota, which may contribute to the production of metabolites with

antimicrobial activity, are not fully implemented. Given the wealth of multi-omics information on commensal microbiota, which is already collected and available for analysis, it is expectedly one of the main avenues to explore in the search for novel antimicrobial drugs.

4.3. Plants

Medicinal plants have been in the arsenal of traditional medicine for millennia, but the identity of compounds that are responsible for therapeutic activities remained poorly defined. One of the well-known examples of a plant-derived antimicrobial is artemisinin, which was initially isolated from sweet wormwood (*Artemisia annua*), the herb used in traditional Chinese medicine [57]. Artemisinin and its derivatives are now used in combination therapies against malaria and other parasitic infections. Another herb, used in traditional Chinese medicine, is *Aquilegia oxysepala*, extract of which exhibits anti-staphylococcal activity [58]. A number of metabolites isolated from the plant extract demonstrated some similarity with antimicrobials with the known mode of action (MoA). More than 400 compounds have been isolated from different parts of the neem tree (*Azadirachta indica*), which is widely used in traditional medicine in Asia for its antimicrobial, antimalarial, antiviral, anti-inflammatory, and many other beneficial properties [59]. Some secondary bioactive metabolites include azadirachtin, nimbidin, nimbin, nimbolide, gedunin, and others.

The plant metabolome is considered to be one of the richest sources of secondary metabolites [60]. In a recent review, the use of metabolomics in plant-based antimicrobial drug discovery has been extensively discussed, with all steps involved, from sample preparation to metabolomics screening to bioinformatics and statistical analysis, and finally to determination of MoA [61]. There is already a substantial number of plant-derived compounds, the mechanisms of antimicrobial action of which are already confirmed by different omics techniques [62]. More extensive use of metabolic techniques to cover metabolites from a broader range of plants, which are not necessarily used in traditional medicine, could potentially contribute to the continuation of the successful trend of identification and characterization of antimicrobial compounds from plants.

4.4. Marine ecosystems

One of the promising ecosystems to explore in the search for novel antimicrobial agents are the marine ecosystems [63,64]. High-throughput metabolomics techniques allow to explore the vast diversity of chemical compounds from marine sources much more rapidly and efficiently compared to the classical bioassay approach [65]. Another point to address here is that compared to the Bacteria, the eukaryotic domain of life retained a limited diversity of secondary metabolites (except plants and fungi) and the production of these metabolites in, for example, marine invertebrates is performed mainly by the associated symbiotic microbiome. A large number of secondary metabolites and bioactive compounds are produced by the symbiotic microbiota of marine invertebrates, but these are difficult to access via the classical bioassay methods due to cultivation difficulties, scarcity of samples, and low concentrations of metabolites produced. Thus, there is a need for advanced metabolomics tools to access this metabolic diversity.

Sponges, for example, cannot produce functional mucus layers and the protective and other functions such as nutritional are performed by symbiotic microbiota, which may comprise up to one-third of the sponge's biomass [66]. The cultivated and molecular diversity of sponge-associated microbiome differs significantly [67]. Thus, although the sponge-associated microbiota produces a wide range of antimicrobial compounds [68,69], obstacles in trying to cultivate these symbionts make it difficult to evaluate this metabolite pool by activity-based screening methods. Thus, the mechanism-based and highly sensitive metabolomic tools, which can operate with small sample sizes to limit sampling damage, could be the most appropriate approach to investigate secondary metabolites produced by these symbiotic consortia. Gene clusters involved in the production of a metabolite of interest could be then accessed via further metagenomic analysis.

Another group of marine invertebrates with a strong symbiotic microbial association and the ability to produce a wide range of bioactive metabolites, including antimicrobials, are the tunicates [70–72]. Bacteria associated with deep-sea coral ecosystems are able to produce antimicrobial compounds as well [73].

At the same time, a wide variety of antimicrobial secondary metabolites are also produced by diverse groups of bacteria associated with other marine invertebrates such as mollusks or residing in other marine environments such as marine sediment, seawater, and marine flora [74]. Various specifics of marine metabolomics have been recently discussed in a review, which illustrated a complete workflow in metabolomics of marine organisms including sample collection, metabolite extraction methods, analytical techniques, data analysis, and dereplication [75]. Applications of metabolomics in marine ecology and drug discovery were also demonstrated. Research in marine natural products is supported by several specialized databases such as MarinLit by RCS [https://marinlit.rsc.org, accessed on 14 July 2022], Dictionary of Marine Natural Products [https://dmnp.chemnetbase.com, accessed on 14 July 2022], MarinChem3D [http://mc3d.qnlm.ac, accessed on 14 July 2022], and the Comprehensive Marine Natural Products Database [76, https://cmnpd.org, accessed on 14 July 2022].

4.5. Synthetic compounds

Search for novel antimicrobials could be also performed via complete synthetic routes, which has been pioneered with the development of sulfonamides such as Prontosil during the early years of the antibiotic era in the 1930s. Considering the extensive body of knowledge, which is now accumulated in the areas of bacterial genomics, transcriptomics, proteomics, and metabolomics, and also the availability of a large variety

of wet and dry lab tools, we are in a much better position to explore this route to identify and characterize new potential targets and drugs.

High-throughput analysis and functional annotation of chemical compounds to reveal novel antimicrobial activities include, for example, combination of metabolomics and gene inactivation data [77,78]. Additional advantage of the synthetic approach is that the drug leads could be optimized to target a narrow range of pathogens or even a single pathogen. The use of broad-spectrum antimicrobials involves a broader range of bacterial populations under selection thus increasing the chances for a corresponding resistance mechanism to emerge. Narrow-spectrum antimicrobials operate at the lower bacterial population numbers thus decreasing these chances. Besides, since narrow-spectrum antimicrobials are more specific toward a limited number of targets, the major constituents of commensal microbiota would not be affected thus preserving its integrity, without plummeting into a dysbiotic state, which is usually a consequence of treatment by broad-spectrum antimicrobials.

4.6. Improvement of known compounds

The history of antimicrobials is a continuous arm race between the microorganisms, which would acquire resistance, sooner or later, after the introduction of a new antimicrobial, and humans trying to come up with a modified version of an antimicrobial that can elude these resistance mechanisms. All major classes of antimicrobials have already been subjected to several cycles of modification to improve their therapeutic properties to counteract the growing problem of antimicrobial resistance among pathogens. Development of new generation antimicrobials usually includes risk assessment of potential resistance emergence. This assessment, however, has certain limitations as it is performed with pure bacterial cultures and could detect only mutational events. The majority of antimicrobial resistance mechanisms are due to the acquired resistance, which is transferred horizontally from other microbiota. These risks should also be evaluated. Besides, based on our current knowledge, it is possible to predict the emergence and dissemination of resistance toward some novel antimicrobials, in particular, if older-generation antimicrobials are able to select for resistance toward newer-generation antimicrobials [79].

Another strategy for reusing the known antimicrobials is to design constructs, which combine two or more pharmacophores, such as a rifamycin-quinolone hybrid drug [80], chimeric streptogramin-tyrocidine [81], a rifamycin-nitroimidazole conjugate [82], kanglemycin-fluoroquinolone hybrids [83], and a number of other hybrid constructs containing different antimicrobial pharmacophores [84]. The known antimicrobial peptides can also be improved through genetic engineering, high-throughput screening, and chemical modifications [85].

5. Metabolomics in drug–target interaction

Elucidation of drug–target interaction is an important step toward understanding of MoA as well as evaluation of potential mechanisms of resistance. It is especially true for novel drugs or for optimization of drug leads in order to improve their pharmacokinetic and pharmacodynamic properties and increase the chances of commercialization [86]. Various high-throughput technologies are extensively used for this purpose. Metabolomic approaches could be especially useful during the earlier stages of antimicrobial drug development in order to identify pathways affected for further identification of molecular target(s). In this area, Zampieri and others [87] developed a rapid high-throughput systematic metabolome profiling strategy to classify MoA of known reference compounds on the metabolome of *Mycobacterium smegmatis*. They then applied this strategy to a library of 212 new anti-mycobacterial compounds with unknown MoAs. More than 70% of these compounds expressed metabolome responses similar to the drugs with known MoAs, while 8% appeared to affect new targets. This approach is a very useful tool in antimicrobial drug discovery programs since it allows to implement a different, mechanism-based, discovery strategy instead of the classical activity-based screening. Thus, this high-throughput mechanism-based strategy significantly accelerates dereplication and facilitates identification and characterization of antimicrobial compounds with novel targets and MoAs.

Recently, a GC-GC-TOFMS approach has been used to identify the effect of decoquinate derivative RMB041 on the metabolome of *Mycobacterium tuberculosis* [88]. On the basis of metabolomic analysis, the authors concluded that the primary target of this drug is the cell wall and secondary – DNA metabolism. Revealing the mechanisms of drug–target interaction is especially interesting in cases of antimicrobials, resistance to which is not easily obtainable under laboratory conditions. One of these drugs is the cyclic depsipeptide teixobactin, and untargeted metabolomics was used to reveal its molecular targets in methicillin-resistant *Staphylococcus aureus* (MRSA) [89]. In the time-dependent antibacterial killing assay, the initial targets were bacterial membrane lipids and cell wall biosynthesis, followed by perturbations of pathways involved in metabolism of amino-sugar, nucleotide-sugar, arginine, tricarboxylic acid cycle, histidine, pantothenate, and coenzyme A.

Metabolomics-based analyses also revealed that bactericidal antibiotics induce a similar set of metabolic changes, even if they belong to different classes and directed toward different bacterial targets [90]. The effects of beta-lactam, aminoglycoside, and quinolone antimicrobials included elevated levels of central carbon metabolites, degradation of nucleotides, reduction of lipids, and elevated redox state. Thus, besides affecting the primary targets, bactericidal antimicrobials also cause metabolic changes leading to the accumulation of toxic metabolites and cellular damage. It remains to be established what are the chains of reactions leading from differential primary drug–target interactions to the similar metabolic effects. Alternative explanations could also be considered such as whether it is a single antimicrobial drug–target interaction, a 'magic bullet,' or the interaction of antimicrobials with the cellular machinery is more complex and potentially include more than a single target [88].

Synergistic action of antimicrobials allows to decrease the effective concentration of antimicrobials thus lowering

potential side effects, while the combination of antimicrobials may limit the emergence of resistance compared to monotherapy. Metabolomic analysis may help to identify pathways that are affected by antimicrobial combinations and reveal the mechanisms of synergy in such combinations. For example, untargeted metabolomics was used to reveal the synergy between colistin and doripenem in killing multidrug-resistant *Acinetobacter baumannii* [91]. The authors found that colistin acts earlier and disrupts the outer membrane and cell wall, while the action of doripenem affects metabolites involved in peptidoglycan biosynthesis. The combination of drugs affected more key metabolic pathways than either of the drugs alone, resulting in depletion of metabolites involved in energy, nucleotide, amino sugar and peptide metabolism.

Another application of metabolomics in drug–target interaction is discovery of new compounds that are not necessarily antimicrobials but can enhance the action of known antimicrobials and thus exert synergistic effect if combined. These compounds can be identified and characterized via monitoring their effect on the metabolome of a pathogen. High-throughput metabolomics profiling of bacterial pathogens in response to a collection of small molecules can significantly facilitate exploring novel antimicrobial compounds and leads compared to the classical approach with time- and labor-consuming experiments based on growth inhibition *in vitro* [77]. These authors performed metabolic profiling of *Escherichia coli* response to 1,279 FDA-approved drugs from the Prestwick Chemical Library and found that a large number of bacterial metabolites are affected by human-targeted drugs, which are not actually antimicrobials. Combining metabolomics with the chemogenomic data, they demonstrated epistatic drug interactions and suggested that this approach could be used in new antimicrobial treatments, which include compounds nonlethal to bacteria. Other potential areas of application could be drug tolerance, side effects, and drug repurposing. In the follow-up work, these authors combined metabolomics and CRISPR interference to design a strategy for comprehensive high-throughput analysis and functional annotation of chemical compound libraries [78]. This strategy can be applied toward a variety of targets, from bacteria to human cell lines. The high-throughput metabolomics approach, for example, was successfully used for predicting drug–target relationships in yeast, *Saccharomyces cerevisiae*, which was treated with the same compounds from the Prestwick Chemical Library, with further target identification through metabolic profiling [92].

There is also a growing interest in exploring of other, non-lethal, bacterial targets such as virulence, biofilm formation, and quorum sensing. For example, it was discovered that clinical strains of *Pseudomonas aeruginosa*, which differ in their virulence and biofilm phenotypes, were also different metabolically [93]. Moreover, metabotypes of *P. aeruginosa* correlated with antimicrobial resistance and clinical outcome in cystic fibrosis patients [94]. Potentially, intervention into the metabolome of a pathogen, via drugs targeting the corresponding metabolic pathways, can open a possibility to modulate its antimicrobial resistance, virulence, and subsequently the severity of infectious disease, especially those that are chronic and require long-term maintenance such as cystic fibrosis. Presently, however, we have a rather limited understanding of the relationship between the metabolome and virulence and antimicrobial resistance in pathogens.

There are a number of mathematical and computational models to analyze various pharmacokinetic and pharmacodynamic properties of drugs [95–98]. The advantage of one of them, vCOMBAT [98] is in the interactive nature of this web-based tool [https://combat-bacteria.org, accessed on 14 July 2022], which allows non-specialists to generate and visualize their own computational models to simulate drug–target binding and drug effects on bacteria.

6. Expert opinion

6.1. Metabolomics: instrumentation

Development of metabolomics in recent years has been largely driven by the improvements in hardware technology and software support (Figure 1). Developments in MS and NMR instrumentation were geared toward technologies, which are more sensitive, can perform more rapid analyses, cover broader range of metabolites, use smaller sample sizes, have high-throughput capabilities, and can be miniaturized and automated. These improvements are especially important for antimicrobial drug discovery programs that explore metabolomics of ecosystems, sampling of which is biomass-restricted, cultivation of their constituents in laboratory represent a major challenge, and metabolites of which present at low concentrations. Metabolomic studies of pharmacokinetics and pharmacodynamics of drugs would also benefit from the development of portable metabolomics platforms, especially in the areas that require medical point-of-care applications. The possibility of further miniaturization and portability would open new opportunities for *in situ* analyses of metabolites in antimicrobial drug discovery programs that explore distant and hard-to-reach ecological systems. Extended sample storage times in these cases, especially if storage conditions are suboptimal, may result in degradation or transformation of certain fragile metabolites, thus skewing the factual metabolomic profiles. Another point in metabolomics research is a cost consideration. A contemporary GC-MS system may cost US $200k–600k, an LC-MS system may cost US $300k–800k depending on configuration, and an NMR system may go up to US $800k [99]. Implementation of sensitive, fast, high-throughput, and automated analyses would help to decrease the cost of analysis per sample. The large increase in a number of MS and NMR spectra, in compatible formats, in public databases would be also of help in identifying metabolites in complex metabolomes or obtained via untargeted metabolomics.

6.2. Metabolomics: bioinformatics

A large number of bioinformatics software and databases are currently available to support analysis of data collected, including preprocessing, feature detection, retention time correction, alignment, annotation, and statistical analyses. Despite that MS and NMR hardware platforms are supplied

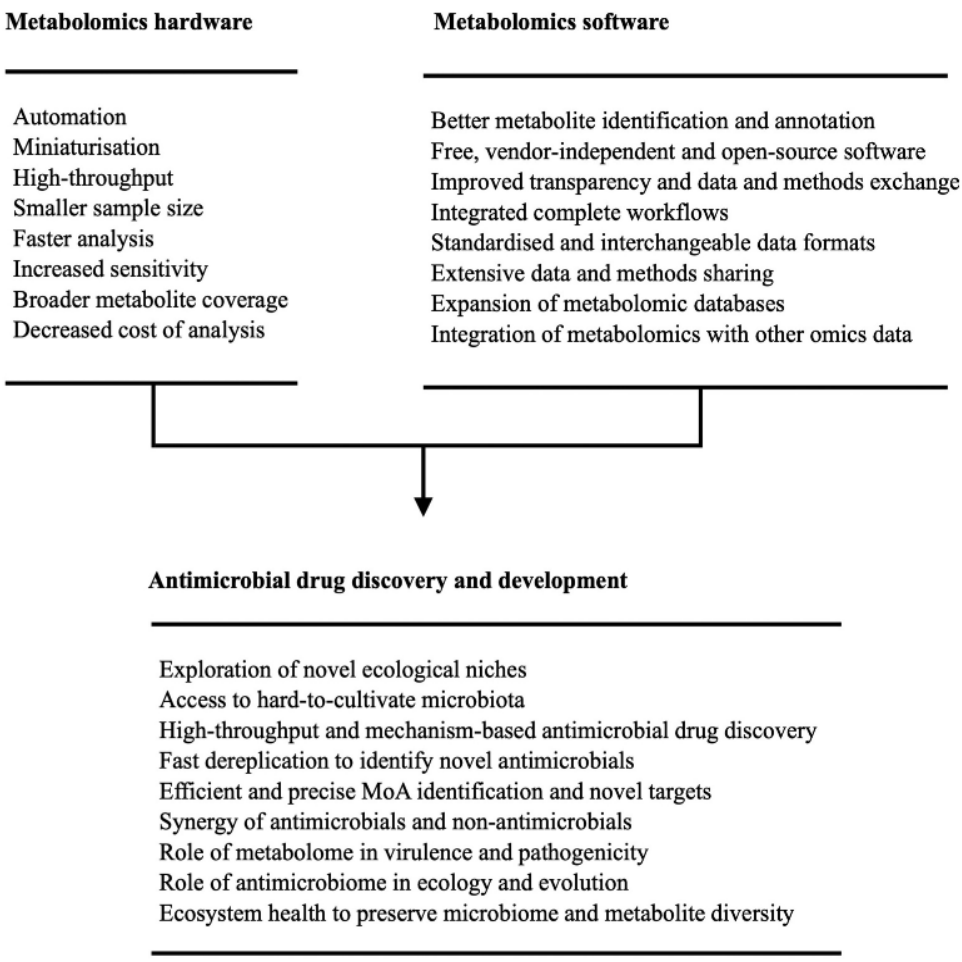

Figure 1. Developments in metabolomics contributing to antimicrobial drug discovery.

by supporting software from the instrument manufacturers and there is also a number of third-party commercial software available, the mainstream development in the software support for metabolomics is the implementation of freely available, open-source, and vendor-independent software (Figure 1). These developments benefit the metabolomics research community, allowing them to work in a vendor-neutral environment, with improved transparency, standardized data formats and reporting, and expedited data and methods sharing.

Another priority is the development of freely available, open-source, and automated software platforms that can provide the complete workflow for high-throughput data, from pre-processing of raw data collected from MS and NMR instruments to identification of metabolites and metabolic pathways. Ideally, these workflows can be integrated into the network of open-access spectral and chemical databases, with a possibility of immediate data sharing. Open data and data mining is a major factor for accelerated discovery and application of natural products and metabolites. Continuous expansion of metabolite databases also facilitates the application of untargeted metabolomics, which is important for the discovery of novel antimicrobial drugs with new targets.

Integration of metabolomics with other omics data is crucial for understanding the structure-and-function of biological systems (systems biology), from a single cell to an organism to a population to an ecosystem. The integrated layers of biological information are important not only for understanding fundamental biological processes but also for a variety of biotechnological and clinical applications such as antimicrobial drug discovery. Finding a metabolite with a potential antimicrobial activity initiates a plethora of works toward genetics, regulation and metabolic pathways for its production, clarification of its biological role and identification of target(s), effects on pathogens and hosts, its pharmacokinetic and pharmacodynamic properties and many other aspects of drug development. Integration of metabolomics data within the multi-omics space, therefore, allows better understanding the function of metabolites within a broader systems biology context. Important roles in this integration are played by meta-analyses and network-based approaches. Another *in silico* approach, which could be a major contributor to antimicrobial drug discovery and development at the large dataset age, is artificial intelligence (AI) [100]. Several AI platforms were developed that are capable of handling the flow of large data streams and help with drug–target identification, lead generation, and lead optimization.

6.3. Metabolomics: antimicrobial drug discovery

The introduction of highly sensitive high-throughput metabolomic techniques opens completely new avenues in antimicrobial drug discovery compared to classical assay methods. The shift from activity-based to mechanism-based metabolomics screening techniques allows analysis of samples, which are difficult to cultivate, present in small quantities, and produce low concentration of metabolites that may not reach lethal concentrations necessary for classical tests (Figure 1). Thus, more novel sources of bioactive compounds and drug leads can be explored compared to the classical screening techniques that require a suspected antimicrobial producer to be present in high concentrations in pure culture to produce a sufficient amount of antimicrobial(s) to exert a killing effect on an indicator strain. The switch from the bottom-up to top-down approach allows scaling up antimicrobial drug discovery efforts and analyze not only pure cultures but also consortia or even whole ecosystems to identify a promising lead.

Another challenge that faces antimicrobial drug discovery programs is the redundancy and re-isolation of producers of the same antimicrobials, which has frequently been the case in the years following the initial golden years of the antimicrobial era. Metabolomic approach, which allows fast dereplication of antimicrobials from natural and synthetic sources, is a powerful tool to make the drug discovery process faster and potentially identify compounds with novel MoAs (Figure 1).

6.4. Metabolomics: target identification

A clear understanding of the MoA of natural and synthetic antimicrobial compounds is crucial for drug characterization and for evaluation of its potential for future development and commercialization. In the previous drug discovery programs, very few drug leads have been subjected to this analysis, because it is labor- and time-consuming. It is usually performed using experimental bacteriology approaches, with a variety of microscopic techniques, by monitoring various biosynthetic processes in the cell in response to antimicrobials, and by gene knockout and overexpression techniques. Then, the drug–target interaction is confirmed by X-ray crystallography using a drug and a purified target. In recent years, this arsenal has been supplemented by whole-genome sequencing and transcriptome and proteome analyses, which accelerated the process of MoA identification. The progress in high-throughput metabolomics, in combination with chemogenomics, gene inactivation, and metabolic effect of antimicrobials with known MoA, now allows a rapid identification of MoAs (Figure 1). The high-throughput nature of this approach allows to perform a large-scale identification of MoAs immediately in the beginning of the drug discovery process, by analyzing the complete libraries of small-molecule drug leads. High-throughput metabolomics also contribute to a faster dereplication process and to the discovery of antimicrobial compounds with novel MoAs.

Important is the discovery of the relationship between the metabolome and pathogenic properties such as virulence, clinical outcome, antimicrobial resistance, and biofilm formation in *P. aeruginosa* [93,94]. This discovery leads to a new field of research, which should provide mechanistic explanation for the phenomenon observed. Currently, it is not clear if the virulence-metabolome link is common for other pathogenic microbiota as well. If so, this opens the prospects of suppressing pathogenic properties of infectious bacterial agents via metabolome modulation and thus limits damage to the host. This approach could be is especially important for the management of chronic bacterial infections that are difficult to control and eradicate.

The discovery of metabolic effects of non-antimicrobials on bacteria and yeast [77,78,92] notably broadened our understanding of drug–target and drug–drug interactions. This progress would be beneficial for drug repurposing efforts and contribute to the development of combinational therapies that include non-lethal drugs that provide synergistic effects to the existing antimicrobials. Another application could be the construction of hybrid drugs [84] that include a non-antimicrobial pharmacophore component, which may enhance the effect of antimicrobial pharmacophore(s).

6.5. Metabolomics; Ecology and evolution

One of the important fundamental questions regarding the role of antimicrobials in microbial ecological niches is what are their actual roles? Derived from the use of antimicrobials as therapeutic agents at killing concentrations, we tend to be under the impression that antimicrobials perform the same function in natural ecosystems, with antimicrobial producers killing their neighbors to protect and extend their own ecological niche. The situation could be more complex, however, if the level of antimicrobial production in natural ecosystems is not reaching killing concentrations. Then, the low-concentration antimicrobials may serve as a language of communication in microbial ecosystems [101, 102], and not only in microbial ecosystems, antimicrobials may have significant regulatory effects on eukaryotic organisms as well [103]. Laboratory experiments with sub-inhibitory concentrations of antimicrobials suggest that the signaling role of antimicrobials at low concentrations may indeed be the case in natural ecosystems. With the advent of more sensitive and portable metabolomics instrumentation, we may now address these questions *in situ*, by measuring the actual levels of various compounds, which display antimicrobial activity at high concentrations but may have regulatory roles at low concentrations, in a variety of ecosystems (Figure 1).

Metabolomics can also help in assessing the important issue of the health and function of natural ecosystems, especially under the current conditions of global climate change. One of the consequences of this change is ocean warming, which impacts various marine ecosystems including coral reefs, with extensive coral bleaching and mortality. Using untargeted metabolomics, Roach and others [104] investigated severe coral bleaching events and concluded that metabolomics can predict the bleaching phenotype with 100% accuracy suggesting cardinal changes in the coral reef metabolome due to the bleaching events. In sponges, elevated temperatures cause stress response in the host and symbiotic microbiota, resulting in the reduction of symbiotic functions

[105]. This ultimately leads to the loss of archetypal sponge symbionts, and opportunistic scavengers start to dominate necrotic sponges. In marine invertebrates, it is the symbiotic microbiota, which is almost exclusively responsible for the production of various bioactive compounds, and the loss of symbionts will inevitably lead to the reduced diversity of important, both for the ecosystems and potential applications, metabolites in marine ecosystems. Increased temperatures also affect soil metabolome, with the increase of labile metabolites and shift toward acetoclastic and methanogenic conditions [106]. Presently, it is difficult to interpret how these changes in soil ecosystems are relevant to the production of secondary metabolites, including antimicrobials, by soil microbiota. Further research is needed to reveal the impact of natural and anthropogenic factors on the diversity of secondary metabolites in natural ecosystems.

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