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Ambient ionization strategies for the characterization of microbial systems *via* mass spectrometry

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Ambient ionization sources enable analysis *via* mass spectrometry (MS) with minimal sample handling and without vacuum-based ionization/sampling. The versatile nature of ambient ionization techniques makes them well suited for both high-throughput analyses and *in situ* spatial characterization. Ambient MS platforms such as desorption electrospray ionization (DESI-MS), direct analysis in real time (DART-MS), paper spray (PS-MS), and secondary electrospray ionization (SESI-MS) are particularly amenable for microbial analysis and have recently been utilized for rapid profiling of microorganisms and imaging of fragile substrates with complex biochemistries. This minireview aims to provide an overview of contemporary ambient ionization technologies coupled with MS and summarize the recent application areas of these strategies in the characterization of microbial systems *via* mass spectrometry over the past five years.

1. Introduction

While mass spectrometry (MS) is concerned with the transfer and analysis of ions in the gas phase, ionization techniques serve as bridging technologies between condensed-phase samples and gas-phase ions. For much of the history of MS, analyses were largely limited to the investigation of volatile chemical species, and it was not until the advent of techniques such as secondary-ion MS (SIMS) in the 1940s and later electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) in the late 1980s that the scope of MS expanded to the study of ions produced from liquid- and solid-phase biological samples, respectively. Ion source development was then again expanded in 2004 with the first description of desorption electrospray ionization (DESI), which enabled the generation of gas-phase analyte ions under ambient conditions from non-volatile materials.[1] DESI is a hybrid, soft-ionization technique that combines the benefits of traditional electrospray methods with the analysis of solid-phase samples. DESI is performed at ambient pressure and requires little to no sample preparation. The introduction of DESI is widely regarded as initiating a new era of ion source development. Since the introduction of DESI more than 40 ambient ionization techniques have been developed and applied in various pharmaceutical, forensic, and biochemical applications—notable examples of these technologies include direct analysis in real time (DART), paper spray ionization (PS), secondary electrospray ionization (SESI), rapid evaporative ionization mass spectrometry (REIMS), and matrix-assisted laser desorption electrospray ionization (MALDESI).[2–6] In the last five years, nearly 1400 peer-reviewed research articles have been published utilizing ambient ionization MS, with more than 150 being published in the first half of 2025 alone (Fig. 1A). The top fifteen most frequently implemented ambient ionization techniques in the last five years are outlined in Table 1.

Due to their integral roles in clinical, environmental, and agricultural contexts, an increasingly important application space for sensitive and high-throughput techniques such as mass spectrometry is in the analysis of microbial samples, including fungi, algae, and prokaryotes. Factors including the advent of technologies such as CRISPR-Cas9 and the burgeoning threat of antibiotic resistance have placed an impetus on biochemists and clinicians to utilize advanced analytical techniques such as MS and on analytical chemists to develop workflows amenable to the demands of modern microbiology. For example, metabolic engineering and synthetic biology strategies offer a viable pathway for developing microbes able to sustainably produce commodity chemicals; however, delineating the effects of individual edits on broad-scale microbial metabolism remains difficult.[37–39] Likewise, while the ability to mine microbial genomes using machine learning (ML) and *in silico* databases for the potential elucidation of novel natural products shows promise, rapid and high-throughput bioanalytical workflows are required to realize and validate these discoveries.[40–42] Furthermore, there is a constant demand for sensitive and reproducible point-of-care and non-proximal technologies able to readily provide microbial characterization

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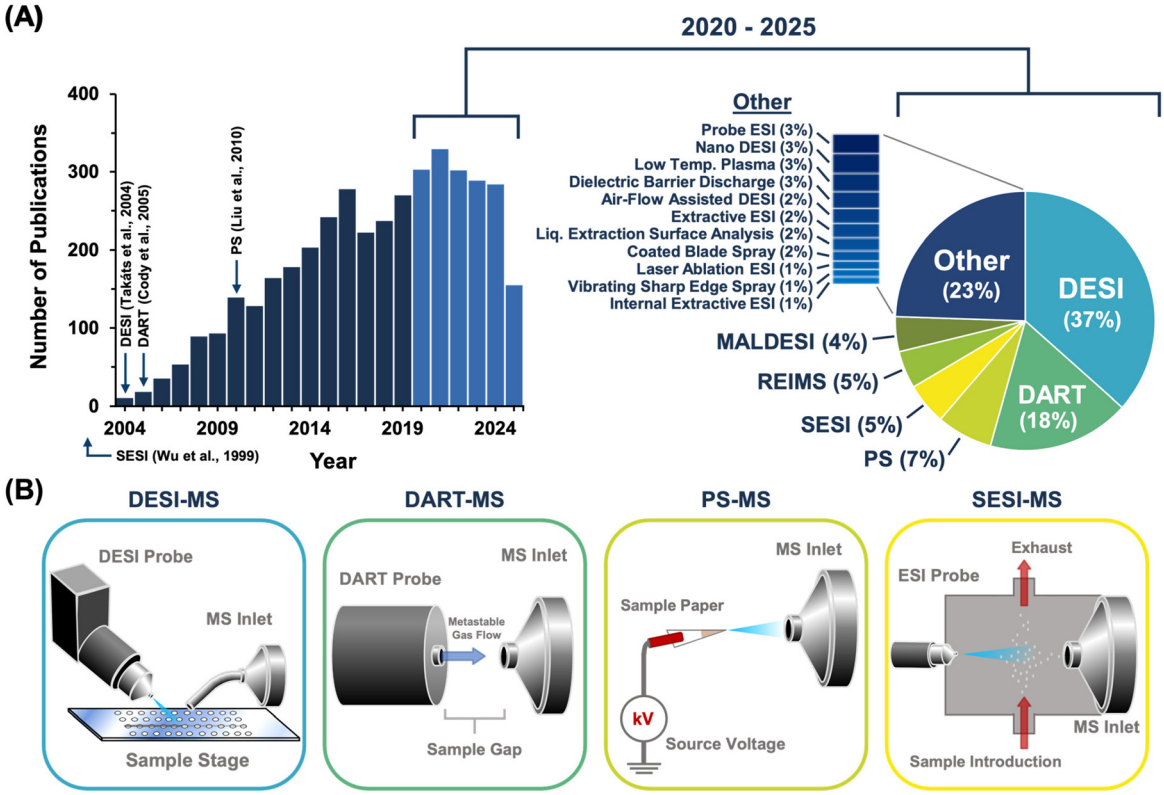

Fig. 1 (A) Histogram of the number of publications published per year implementing ambient ionization mass spectrometry (left) and representative contribution for individual ambient techniques based on the number of publications in the last five years (right). The 15 most frequently utilized ionization sources are outlined in Table 1. (B) Graphical representations of the top four most frequently implemented mass spectrometry (MS) ionization techniques: desorption electrospray ionization-MS (DESI-MS), direct analysis in real time-MS (DART-MS), paper spray ionization-MS (PS-MS), and secondary electrospray ionization-MS (SESI-MS).

Table 1 Most frequently implemented ambient ionization sources in last five years

Ambient technique	Acronym	Desorption method	Ionization method	Spatially resolved capabilities? (y/n)	Ref.
Desorption electrospray ionization	DESI	Spray-based extraction	Electrospray	y	1, 7–15
Direct analysis in real time	DART	Plasma desorption	Corona discharge	n	2, 16–20
Paper spray ionization	PS	Substrate spray	Electrospray	n	3 and 21
Secondary electrospray ionization	SESI	Spray-based extraction	Electrospray	n	4, 22–26
Rapid evaporative ionization mass spectrometry	REIMS	Thermal desorption	Thermal evaporation	n	5
Matrix-assisted laser desorption electrospray ionization	MALDESI	Laser ablation	Electrospray	y	6
Probe electrospray ionization	PESI	Substrate spray	Electrospray	y	27
Nano-desorption electrospray ionization	Nano-DESI	Direct liquid extraction	Electrospray	y	28
Low-temperature plasma	LTP	Plasma desorption	Dielectric barrier discharge	y	29
Dielectric barrier discharge ionization	DBDI	Plasma desorption	Dielectric barrier discharge	y	30
Air-flow-assisted desorption electrospray ionization	AFADESI	Spray-based extraction	Electrospray	y	31
Extractive electrospray ionization	EESI	Spray-based extraction	Electrospray	n	32 and 33
Liquid extraction surface analysis	LESA	Direct liquid extraction	Electrospray	y	34
Coated blade spray	CBS	Substrate spray	Electrospray	n	35
Laser ablation electrospray ionization	LAESI	Laser ablation	Electrospray	y	36

beyond laboratory settings. Conventional spectroscopic methods, such as Fourier-transform infrared (FTIR) and Raman spectroscopy, can provide label-free microbial identification and non-invasive microbial fingerprinting; however, they are limited in their molecular specificity.[43–45] Techniques such as riboswitches or coupled enzyme reactions can also be incorporated into microbial analyses, but these techniques typically require upstream bioengineering prior to implementation. While metagenomic approaches are able to provide information on microbial composition, additional techniques are required to move beyond taxonomic identification and provide direct phenotypic or functional information based on measured metabolites.[46,47]

MS-based analyses are often implemented in the study of microorganisms, leveraging the high sensitivity and specificity of the technique to provide chemical readouts of small molecules *via* lipidomic and metabolomic workflows. Most of the MS methods used to investigate microbial systems implement some form of upstream chromatography, either gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), to maximize molecular coverage.[48–50] This improved chemical specificity often comes at the cost of throughput, wherein sample handling requirements and lengthy analysis times from the separation are bottlenecks in biochemical strategies. However, ambient ionization MS techniques are particularly well suited for the investigation of microbial systems, as their minimal sample handling requirements allow for direct and *in situ* analyses. Furthermore, they are often compatible with complex matrices and short analysis times which enable them to be used in high-throughput workflows. Additionally, techniques such as DESI allow for spatiochemical analyses *via* MS imaging (MSI), providing additional analytical information to help elucidate the chemical environment of complex biochemical systems. In clinical contexts, ambient ionization strategies are increasingly implemented for rapid pathogen identification, whereas traditional LC-MS approaches may be too slow to inform timely decisions. Some MS workflows implement MALDI-based approaches, as laser desorption provides superior spatial resolution and more reproducible signal considerations, but matrix interferences and compatibility issues associated with the requirement for vacuum-based sampling can hinder the analysis of more labile and volatile microbial analytes. In environmental and agricultural microbiology, ambient techniques are increasingly being used for the spatial characterization of host–pathogen interactions and in field-deployable platforms for remote analyses.

In the present minireview, we focus on the four most frequently implemented ambient techniques over the last five years, DESI, DART, PS, and SESI, and their applications in the MS analysis of microbial systems (Fig. 1B). We provide an overview of ambient ionization strategies and outline their implementation in microbial identification, screening, and metabolic profiling. Finally, we summarize the current challenges and offer a perspective on the future direction of ambient MS for microbial systems.

2. Overview of ambient ionization techniques

Generally, ambient ionization techniques can be characterized by decoupling analyte desorption and ionization. Desorption describes the manner in which the source extracts and releases analyte molecules from the sample matrix in the form of neutral or pre-formed ionized species, while ionization represents the process of subsequent ion formation of neutral molecules and their introduction into a mass spectrometer. The three main desorption categories are (1) liquid extraction, (2) laser ablation, and (3) plasma desorption.

Liquid extraction-based approaches utilize a solvent to extract molecules from the matrix at the sample surface. Broadly speaking, there are three main categories of liquid extractive techniques: (i) spray-based extraction, (ii) direct liquid extraction, and (iii) substrate spray. Spray-based extractions, typified by DESI, incorporate a solvent spray directed at a sample in order to extract and desorb materials from a surface. In the case of DESI and air-flow-assisted desorption electrospray ionization (AFADESI), the solvent spray is typically directed at a solid surface, with some techniques such as extractive electrospray ionization (EESI) and SESI providing important variations.[31,32] In EESI, desorption occurs between two separate electrospray plumes, while in SESI, extraction occurs for analytes already desorbed into the gas phase. Once extracted and desorbed, analytes then undergo ionization, generally *via* electrospray (ESI) mechanisms, and are directed into a mass spectrometer for analysis. DESI has been extensively applied to surface analyses and in MSI contexts where spatiochemical interrogation is valued, while SESI is largely utilized in the analysis of volatile and semi-volatile species, as it enables real-time monitoring of vapor-phase analytes.

In contrast, direct liquid extraction provides a solid–liquid phase extraction across a liquid microjunction, wherein the solvent comes into direct contact with the sample surface to extract analytes into the solvent stream directed to the ESI emitter. In the case of nano-DESI, extraction and direction into the nano-ESI emitter occurs at the junction of two angled capillaries, where solvent continuously flows across the microjunction, extracting molecules for introduction into a mass spectrometer.[28] Liquid extraction surface analysis (LESA) represents a slight variation of this method, utilizing a discrete droplet rather than a continuous solvent flow at the microjunction.[34] Substrate spray techniques, while extractive, are unique in that the sample substrate provides the medium for both extraction and ion generation to occur directly. Three common approaches are PS, probe electrospray ionization (PESI), and coated blade spray (CBS) that use paper, needles, and functionalized blades, respectively. All three of these techniques integrate sampling, extraction, and ionization into a single step.[27,35] As such, workflows incorporating PS have been developed, emphasizing low-volume analyses and point-of-care measurements.

Laser ablation is also a common desorptive approach that utilizes a pulsed laser directed at a sample surface to generate a plume of gas-phase molecules. Techniques such as laser ablation electrospray ionization (LAESI) and MALDESI use an ultraviolet or infrared laser for initial desorption and an orthogonal electrospray stream for ionization and introduction into the MS.[6] Ambient techniques that utilize laser ablation desorption are frequently spatially resolved, with the spatial resolution dictated by the dimensions of a pulsed laser at the sample surface. These techniques generally offer higher spatial resolution compared to spatially resolved spray-based extraction/desorption techniques, which are often operated continuously, and thus, spatial resolution is limited by the spot size of the solvent spray, with pixel size determined by user-defined parameters (*e.g.*, spray desorption angle and raster rate).

The third main class of desorption methods, plasma desorption, differs from the previous two in that, instead of solvent or photons interacting directly with the sample surface, it is the excited gas molecules formed through a plasma discharge that lead to sample desorption and ionization *via* corona discharge. Plasma desorption techniques operated under ambient conditions are considered a form of atmospheric-pressure chemical ionization (APCI). The most widely used ambient plasma desorption source is DART, although other plasma-based ambient techniques have also been developed, including low-temperature plasma (LTP) ionization and dielectric barrier discharge ionization (DBDI).[29,30] Importantly, these techniques can be tuned for the analysis of nonpolar analytes, which are typically not ionized *via* solvent- or ESI-based techniques. Plasma desorption techniques tend to be more selective towards ionizing small molecules in comparison with higher molecular-weight species that are readily observed in solvent- or ESI-based approaches. To this end, DART has widely been utilized for the rapid screening of small molecules and semi-volatile compounds, particularly in contexts where rapid and high-throughput methodologies are prioritized.

While these desorption methods represent the most utilized ambient MS strategies, there are other prominent source types for which the mechanistic aspects are more challenging to characterize within these three general categories. For example, REIMS is a prominent ambient ionization technique which uses thermal desorption to volatilize and ionize samples simultaneously.

3. Current applications to microbial characterization

Ambient ionization techniques have been utilized in the metabolic profiling of microbial samples, especially in instances where sample handling requirements of more conventional analysis types have the tendency to obscure chemical profiles due to the complexity of the biological systems being investigated. An example was demonstrated by Ollivier *et al.*, wherein DART-MS was used to characterize the metabolic profiles of various lichen samples, each representing a distinct symbiotic microsystem of species from multiple microbial domains.[16] In this study, DART-MS was used to analyse intact lichen thalli directly, providing readouts for numerous analyte classes including polyphenols, benzenoids, and unsaturated hydrocarbons. DART-MS facilitated the rapid metabolic profiling of lichen which would otherwise be hindered by sample handling or potentially biased *via* solvent extraction. Ambient ionization strategies, particularly DART, are frequently associated with the analysis of volatile organic compounds (VOCs), with the metabolomic investigation of these analytes increasingly being termed volatilomics. In one such study, Busman *et al.* implemented DART-MS for the characterization of fungal VOCs, whereby non-invasive metabolic fingerprinting of plant pathogen species belonging to the *Fusarium* genus was achieved.[17] The use of DART allowed for direct sampling of the headspace of fungal culture bottles, without VOC trapping or chromatography, and provided information on both species-specific ions and species-specific VOC production profiles. The unique characterization profiles have the potential to serve as a basis for rapid species discrimination, with the headspace analysis implemented providing a platform for repeat measurements with minimal perturbation during time-course evaluations.

SESI has also been utilized in the characterization of microbial VOCs, with Mengers *et al.* using an SESI source coupled to an Orbitrap MS for the online and real-time off-gas analysis of yeast fermentation.[22] These time-course studies actively monitored changes in ethanol, acetaldehyde, and other VOCs which reflected the underlying metabolic shifts occurring as the cultures progressed through various growth phases. Subsequently, Choueiry *et al.* developed a SESI-MS-based untargeted volatilomics workflow which was used for the analysis of anaerobic bacterial cultures.[23] Also incorporating headspace-based sampling, the focus of this work was the development of a novel pseudo-targeted approach that worked toward the optimization of VOC annotation to aid in species differentiation. This database-assisted workflow was able to detect an increased number of significantly altered features when compared to conventional data-dependent acquisitions and other targeted methods. Alternatively, DESI-MS has also been used in the monitoring of microbial growth phases, with Szalwinski *et al.* characterizing *Escherichia coli* through the evaluation of lipid profiles rather than VOCs.[7] While the previous applications focused on volatile chemical species, DESI-MS highlights the ability for ambient ionization sources to desorb and ionize higher-molecular-weight species directly from solid-phase samples. In this study, manually deposited bacterial extracts were analysed *via* a two-dimensional tandem MS (MS/MS) workflow, wherein the lipid precursor, fragmentation products, and signal intensities were rapidly evaluated. Sample interrogation revealed fatty acid chain modifications in phosphatidylethanolamine and phosphatidylglycerol lipids over time, allowing for the differentiation of *E. coli* lysates collected during lag, exponential, and stationary growth phases. These observed PE and PG chain modifications are significant

in that fatty acid length and degree of unsaturation are highly regulated chemical attributes that dictate cellular membrane fluidity, rigidity, and shape, which directly impact cellular metabolism.

Studies which garner analytical benefits of solid-phase DESI analyses can leverage the spatially resolved nature of the technique. Microbial imaging *via* DESI mass spectrometry imaging (DESI-MSI) demonstrates the unique benefits of ambient imaging, allowing for *in situ* spatial analyte characterization. In a study by Xu *et al.*, four species of lichen were imaged using DESI-MSI to reveal heterogeneous spatial distributions of the pharmaceutically relevant secondary metabolite usnic acid, which exhibits a wide spectrum of potent bioactivities.[8] DESI images of lichen thalli cross-sections revealed that the antibiotic compound primarily concentrates in cortical hyphae as expected, but usnic acid was also found to be accumulated in the algal vicinity (Fig. 2A). In the same study, which marks the first investigation of lichen *via* DESI-MSI, spatial information was combined with enantiomeric ratios measured by chiral high-performance liquid chromatography coupled to photodiode array detection (HPLC-PDA) to provide a more holistic understanding of usnic acid production, which exists in two chiral forms that exhibit different biological activities. In a related application, Xia *et al.* investigated the spatiochemical distribution of the triterpenoid secondary metabolite ganoderic acid and its related metabolites in the macrofungus *Ganoderma lingzhi*.[9] Characterizing more than 130 different metabolites at four different stages of maturity, the study involved imaging cryosections of various regions of the fungal morphology, wherein components of fatty acid biosynthesis and natural product metabolism were visualized (Fig. 2B). Recently, macrofungal metabolite distributions have also been reported for the ascomycete fungus, *Cordyceps cicadae*, where Cao *et al.* used DESI-MSI to map the spatial orientations of fungal microregions *in situ*.[10]

The spatially resolved nature of DESI-MSI analyses can also be leveraged to aid in addressing the throughput demands of modern synthetic biology. One such workflow by Ellis *et al.* uses the imaging capability of DESI for spatial multiplexing, wherein the phenotypic profiles of multiple strains of genetically engineered bacteria were simultaneously characterized and the desired metabolic product production was evaluated in a single acquisition.[11] Whereas previously described DESI-MSI workflows imaged either cross-sections or cryosections of microbial samples, this spatially multiplexed approach imaged co-cultured strains grown on a nylon membrane *in situ* and differentiated the metabolic signatures using an unsupervised segmentation algorithm (Fig. 2C). Analysis time for microbial screening was decreased significantly due to both reduced sample handling compared to conventional LC-MS and GC-MS workflows and the ability to analyse multiple strains concurrently. This DESI-MSI approach provided untar-

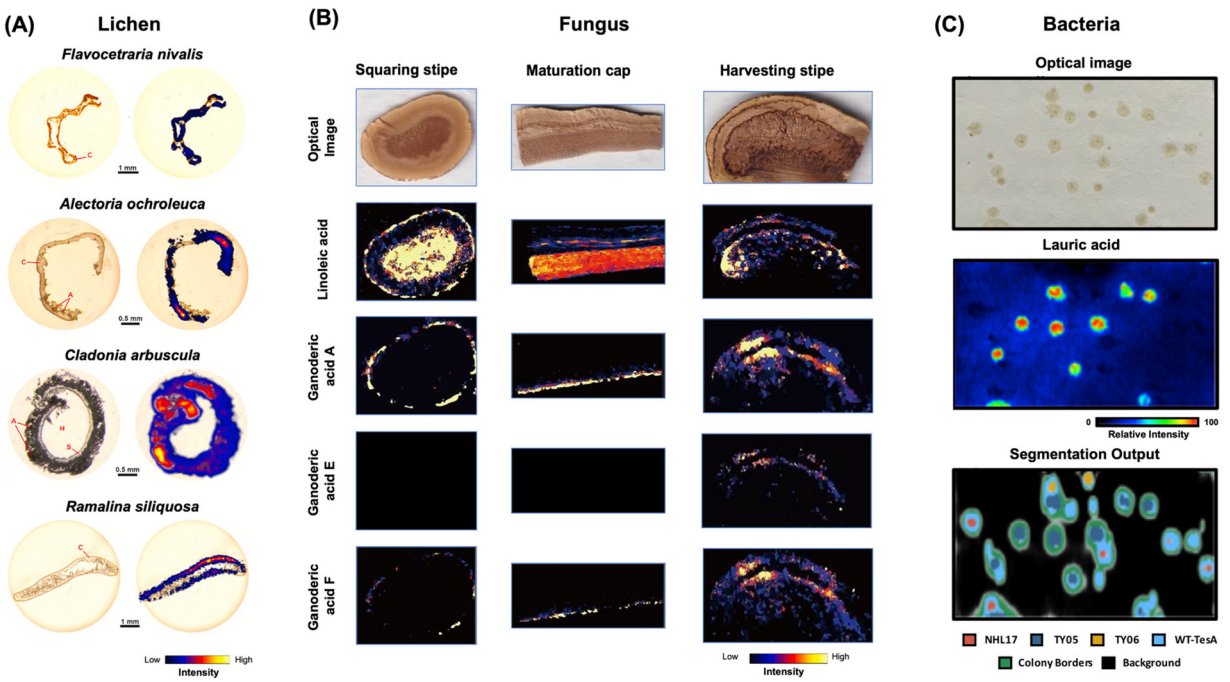

Fig. 2 Several examples of *in situ* spatial analysis using DESI-MSI. (A) Four species of lichen (left column of optical images) were imaged using DESI-MSI to determine the spatial distribution of usnic acid, an antibiotic natural product (right column of molecular images). Adapted with permission from ref. 8. Copyright © 2022 Elsevier. (B) Imaging cryosections of the macrofungus *Ganoderma lingzhi* (top optical images) allowed for the spatiochemical analysis of the natural product distribution of ganoderic acid and its analogues (bottom molecular images). Adapted with permission from ref. 9. Copyright © 2024 Elsevier. (C) Genetically engineered *Escherichia coli*, designed to overproduce free fatty acids, were plated as a co-culture on top of an agar-supported nylon membrane. Spatially multiplexed imaging and unsupervised segmentation allowed for strain differentiation during a single acquisition based on bacterial metabolic profiles. Adapted from ref. 11, licensed under CC BY 4.0.

geted metabolic information at a rate of 8 min per microbial colony and was used to compare the free fatty acid production profiles of various *E. coli* strains containing genetically engineered thioesterase modifications. Recently, Shepard *et al.* further reduced the analysis time associated with screening bioengineered bacteria through the development of a DESI-MSI line-scan workflow for untargeted phenotyping.[12] This "fast-pass" strategy increased throughput by reducing the amount of spatial information required for strain differentiation without forfeiting the depth or quality of analysis. Using this fast-pass approach, global metabolic characterization was obtained at a rate of ~40 seconds per sample.

Other ambient ionization techniques that have been utilized for rapid strain-level differentiation include DART-MS, PS-MS, and SESI-MS. *Legionella pneumophila*, the main causative agent of Legionnaires' disease, was successfully profiled *via* DART-MS in a study reported by Tata *et al.*[18] In this work, not only were *L. pneumophila* samples successfully discriminated from other non-*pneumophila* samples, but also differentiation of serotypes of *L. pneumophila* was demonstrated using supervised machine learning (ML). Analyses were performed in the absence of chromatographic separation or sample derivatization, providing a rapid methodology for profiling the water-based pathogen with high classification accuracy and minimal active sample handling. In a different approach, paper spray ionization was coupled with ion mobility-MS (IM-MS) in a workflow developed by Olajide *et al.* for the rapid discrimination of *E. coli* strains through multivariate analysis of isomeric lipid biomarkers.[21] While the implementation of paper spray inherently decreased the sample handling as no liquid–liquid extraction is required prior to the loading of bacteria onto the paper spray filters, IM provides an additional gas-phase separation that affords a molecular size descriptor for enhanced strain differentiation. This study demonstrated the use of paper spray in the identification of clinically relevant *E. coli* strains and the potential of IM separations in rapid phenotyping workflows.

Another clinically relevant application of ambient ionization in microbial strain differentiation was outlined in a report by Kaeslin *et al.*, where SESI was used for VOC biomarker discovery within the context of bacterial pathogens related to cystic fibrosis (CF).[24] Conventional diagnostic methods for CF frequently require culturing prior to analysis, which can result in long sample preparation and analysis times that delay decision-making and treatment. The SESI-MS/MS methodology described provided the differentiation of six strains of bacterial pathogens *in vitro*, with the study yielding 94 distinctive molecular features and 33 putatively identified biomarkers (Fig. 3A). VOC biomarkers corresponding to pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus* may ultimately provide a platform for future diagnostics. Current developments are aimed toward *in vivo* diagnostic workflows.

Recently, a similar strategy was demonstrated by Arnold *et al.* for the early detection of bacterial pneumonia *in vivo*.[25] In this study, SESI-MS was used to monitor the VOCs exhaled by mice during the course of a lung bacterial infection from human respiratory pathogens. This study identified significant variations in the abundances of 25 potential infection-related chemical species. While the goal of ambient strain differentiation strategies in a clinical setting is to improve patient care by expediting identification and diagnosis, data analysis requirements can pose a barrier to the timely and accurate detection of microbial pathogens. Arora *et al.* sought to address this potential bottleneck through the integration of ML-based classification algorithms in the interpretation of microbial VOC profiles measured by DART-MS.[19] As the success of algorithmic discrimination is highly dependent on both the quantity and the quality of the training set data, ML approaches will continue to improve through increased implementation of ambient techniques and further standardization of ambient MS workflows.

While rapid strain level discrimination based on holistic lipidomic or volatilomic profiles has clear utility in clinical and biotechnological contexts, many microbial applications require moving beyond qualitative taxonomic and phenotypic identification and toward the quantitative analysis of specific analytes. The spatially multiplexed and single-raster screening workflows described above are able to provide comparative readouts for target biosynthetic products such as free fatty acids, but they cannot correlate those production levels to specific concentrations without integrating quantitative calibration procedures into the analyses. Frequently, DESI-MS and DESI-MSI workflows are limited in their quantitative capabilities by low sampling reproducibility, the potentially inherent heterogeneous nature of spatial analyte distribution, and potential difficulty with the effective integration of calibration standards.

The use of DART-MS for quantitative microbial analysis was explored as a method to rapidly measure anatoxin levels in benthic cyanobacterial cultures.[20] In a workflow developed by Beach *et al.*, DART-MS was used to analyse 45 *Microcoleus*-dominated cyanobacterial mats harvested from river bottoms. The levels of three anatoxin structural analogues were detected and quantified, and the results also were consistent with quantitation achieved by a conventional LC-MS reference method (Fig. 3B). In this environmental microbiology application, the DART-MS workflow yielded an estimated limit of detection of 5 ng g^{-1} for the neurotoxin and a throughput of 2 min per triplicate analysis.

A recent and expanding area of interest for MS-based analysis is in the characterization of interspecies symbiosis, specifically host–microbiome interactions. A noteworthy study was conducted by Pruski *et al.*, wherein DESI-MS enabled a sample preparation-free method for the rapid microbial profiling of the vaginal microbiome in pregnant women, providing information concerning both bacterial composition and host inflammatory status (Fig. 3C).[13] Evaluation of microbial species diversity, microbial instability, and discriminatory biomarkers facilitated the assessment of preterm birth risk and the selection of preventative treatments. This is especially important considering the potential effects of microbiota dysregulation on the host immune response during gestation.

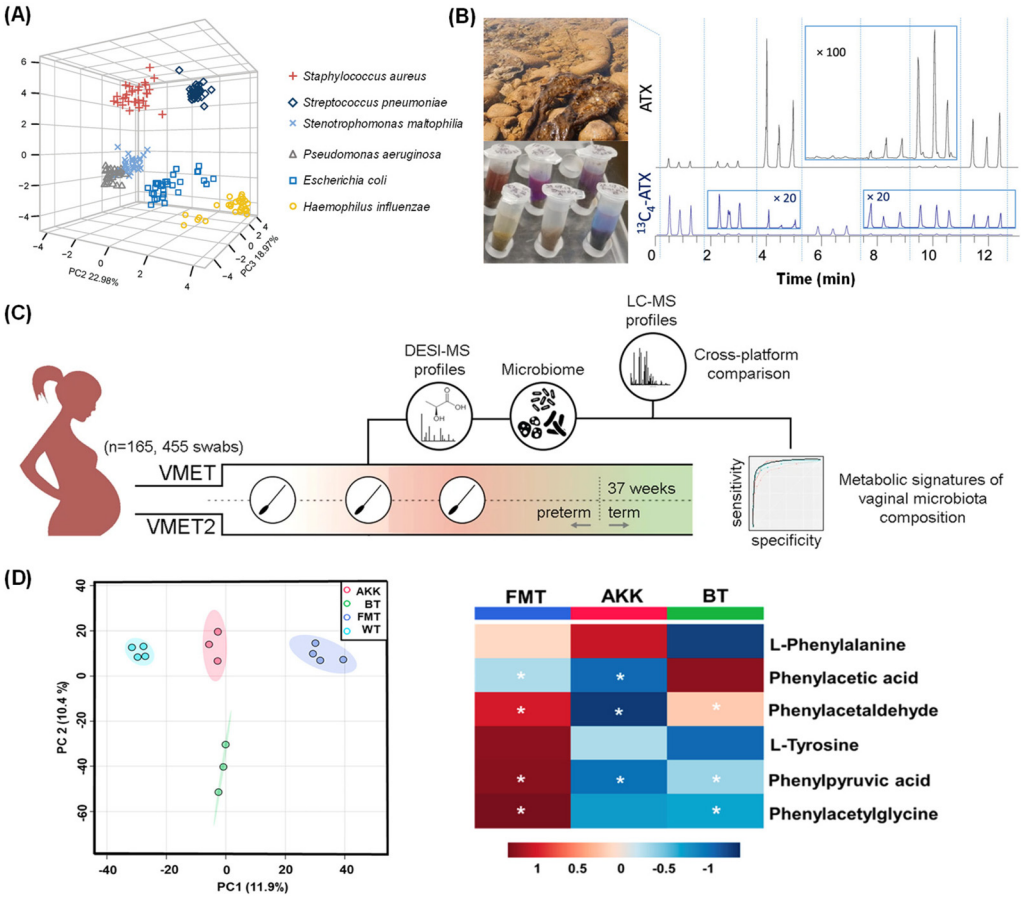

Fig. 3 Examples of ambient ionization workflows applied in the characterization of microbial systems. (A) SESI-MS allowed for the discrimination of six strains of cystic fibrosis-related pathogens, with the differences in their volatilome visualized in a 3-dimensional principal component analysis plot. Adapted with permission from ref. 24, licensed under CC BY 4.0. (B) DART-MS allowed for the rapid quantification of the neurotoxin anatoxin-a (ATX) in cyanobacterial mats sampled from river bottoms. Adapted from ref. 20, licensed under CC BY 4.0. (C) Host–microbiome interactions were monitored in pregnant women *via* DESI-MS, wherein sample preparation-free analyses were able to measure microbial diversity and provide a risk assessment for preterm labor. Adapted from ref. 13, licensed under CC BY 4.0. (D) Intestinal microbiome-dictated changes in metabolism were detected *via* SESI-MS, with three different inoculation conditions distinguished based on volatile compounds exhaled by mice. In this study, wild type mice were compared to mice which had undergone faecal transplantation (FMT), inoculation with *Akkermansia muciniphila* (AKK), and inoculation with *Bacteroides thetaiotaomicron* (BT). Adapted with permission from ref. 26. Copyright © 2023 American Chemical Society.

In a separate study, SESI-MS was used to evaluate how variations in the intestinal microbiome can lead to altered metabolomic and volatilomic profiles of murine host models.[26] In this application described by Choueiry *et al.*, exhaled VOCs were measured directly from inoculated mice using an induction chamber for online sample introduction. Significant metabolic alterations were observed for pathways associated with pyruvate, pyrimidine, and amino acid metabolism, with various inoculation conditions being differentiated based solely on microbiome-dictated VOC signatures (Fig. 3D).

4. Conclusions and future prospects

The application of ambient ionization strategies for microbial characterization is often overshadowed by biomedical applications such as high-spatial-resolution tissue imaging and pharmaceutical compound monitoring. However, the minimal sample handling requirements and potential for rapid analysis make ambient sampling MS methods particularly well suited for broad application in the fields of clinical microbiology, environmental microbiology, synthetic biology, and metabolic engineering. A survey of ambient ionization techniques applied to the characterization of microbial systems using MS over the past five years highlights several analytical trends, including (1) rapid strain differentiation by PS-MS and SESI-MS, (2) *in situ* microbial imaging *via* DESI-MSI, and (3) rapid bacterial volatilomics *via* DART-MS – among others. These advances suggest that new and emerging microbial applications are on the horizon.

As ambient ionization techniques continue to develop, it is expected that complementary analyses including ion mobility separations and non-destructive imaging approaches will be increasingly integrated into ambient workflows to bolster the

chemical information garnered by ambient MS. As chromatography is not feasible for the majority of ambient ionization strategies, one application area where IM separations are positioned to be particularly useful is in ambient ionization imaging workflows such as DESI-MSI. Ion mobility has the potential, in some cases, to serve as a surrogate for chromatography, offering a rapid chemical separation that does not limit throughput while providing an additional molecular descriptor to increase the confidence associated with molecular annotation.[14]

Currently, ambient ionization methods are often viewed as semi-quantitative, largely due to uncontrolled sampling conditions and the effects of matrix variability on ionization efficiency. To this end, we anticipate that future advancements in ambient ionization techniques will include control of the ambient environment (*e.g.*, pressure, temperature, and humidity) to improve upon the achievable figures of merit. There is increasing evidence suggesting that ambient MS results are strongly influenced by factors such as the local humidity present at the site of sampling, potentially affecting sensitivity, reproducibility, and chemical selectivity.[15,33,51] The impact of variation under the conditions of the sampling environment on ambient data is a current need to be addressed, either by the reporting of specific laboratory conditions that provide an analytical context for ambient results or through environmental control systems capable of regulating conditions such as pressure, temperature, and relative humidity to mitigate these sources of analytical variability. Additionally, quantitative strategies incorporating standards spotted both adjacent to and on top of the sampling areas have shown promise for improving the accuracy of such studies.[52,53]

Ion suppression also remains a significant consideration when designing ambient experiments, as ambient conditions and complex microbial matrices can lead to measurable signal variability and reproducibility concerns. While the control systems discussed previously can aid in controlling for environmental variability, the mitigation of signal suppression resulting from bacterial matrices may require some level of sample pre-treatment or sampling surface modification. In some applications, decreased analyte signal arising from high concentrations of inorganic salts present in the sample substrate has been mitigated by either altering the bacterial growth media or incorporating a non-invasive solvent wash.[54,55]

Finally, in tandem with the higher accuracy and precision afforded by ambient ionization techniques, ML-based approaches for data handling in high-throughput screening workflows are expected to become more prominent in terms of both strain differentiation and metabolic interpretation in diagnostics and clinical applications. Microbial systems are also a rich source of pharmaceutically relevant natural products, and the use of ML for sample prioritization will further decrease the timeframe associated with drug discovery and development. As ML techniques continue to become more refined, their application to microbial analyses will expand. ML algorithms such as Convolutional Neural Networks (CNNs) have been developed that can aid in both imaging dataset interpretation and metabolic pathway elucidation. While initially developed for tissue-based and single-cell imaging contexts, these ML tools also have the potential to facilitate both the feature prioritization and the identification of complex spatial patterns in microbial systems.[56–58]

The ambient techniques discussed in this review and their implementation in microbial characterization represent an actively developing application space for MS-based investigations, and we anticipate that this area of inquiry will continue to expand in the foreseeable future with the continued improvement of current techniques and the development of novel ambient sources. New sources such as desorption electro-flow focusing ionization (DEFFI) show promise in mitigating sensitivity issues, while modifications to existing workflows can allow for microbial analyses to be conducted with finer spatial resolution and improved reproducibility.[59,60] These developments will expand the scope of ambient MS investigations not only to new sampling environments but also to broader experimental contexts such as more rapid early detection, real-time host–pathogen interaction monitoring, and deep microbial metabolism elucidation.

Author contributions

This manuscript represents a collaborative effort of all listed authors, each of whom has reviewed and approved the final submission.

Conflicts of interest

There are no conflicts to declare.

Data availability

This review includes no primary research results, software, or code; consequently, no new data were generated or analysed.

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