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Review paper

Modern approaches for detection of volatile organic compounds in metabolic studies focusing on pathogenic bacteria: Current state of the art

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A B S T R A C T

Pathogenic microorganisms produce numerous metabolites, including volatile organic compounds (VOCs). Monitoring these metabolites in biological matrices (e.g., urine, blood, or breath) can reveal the presence of specific microorganisms, enabling the early diagnosis of infections and the timely implementation of targeted therapy. However, complex matrices only contain trace levels of VOCs, and their constituent components can hinder determination of these compounds. Therefore, modern analytical techniques enabling the non-invasive identification and precise quantification of microbial VOCs are needed. In this paper, we discuss bacterial VOC analysis under *in vitro* conditions, in animal models and disease diagnosis in humans, including techniques for offline and online analysis in clinical settings. We also consider the advantages and limitations of novel microextraction techniques used to prepare biological samples for VOC analysis, in addition to reviewing current clinical studies on bacterial volatilomes that address inter-species interactions, the kinetics of VOC metabolism, and species- and drug-resistance specificity.

1. Introduction

Bacterial infections have been re-emerging as a significant threat to patients due to the continued growth of multidrug resistance. According to the European Centre for Disease Prevention and Control's (ECDC) Antimicrobial Resistance Report, a significant increase in antimicrobial resistance to at least one antibiotic group was observed for nearly all monitored bacteria species (except *Escherichia coli* (*E. coli*)) in 2020 [1]. One key approach to limiting the development of antibiotic resistance is to implement an antimicrobial agent specifically suited to the causative pathogen as early as possible rather than employing empiric broad-spectrum therapy. Unfortunately, microbiological testing is time-consuming (minimum of two days), and culturing the microbes requires sample-collection procedures that can be very invasive, such as in the sampling of bronchoalveolar lavage (BAL) for the diagnosis of ventilation-associated pneumonia (VAP). As such, non-invasive methods that enable the rapid identification of infecting microbes (and perhaps their susceptibility to antibiotics) are urgently needed.

The analysis of microbial volatile organic compounds (VOCs) in exhaled breath and human body fluids (urine, blood, and saliva) is a promising clinical approach, as it can enable the diagnosis of bacterial infections in a fast and non-invasive manner [2]. VOCs are mainly secondary products of the aerobic and anaerobic fermentation of bacteria [3], and they represent a diverse group of carbon-based compounds, including carbohydrates, aldehydes, ketones, alcohols, acids, isocyanates, amines, terpenes, and sulfides. Since some VOCs are unique to certain pathogens, they can be used as biomarkers in the identification of specific bacteria. Furthermore, monitoring the profiles of microbial VOCs can enable the detection of emerging infections, which can in turn allow clinicians to implement antimicrobial therapy in a timely manner and monitor its effectiveness, both of which being fundamental components of personalized medicine.

Over the past few decades, significant advances have been made in this area, with findings showing the suitability of direct techniques for the noninvasive diagnosis of bacterial infections based solely on VOC analysis by means of sensor arrays [4–7], gas chromatography-ion mobility spectrometry (GC-IMS) [8,9],

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secondary electrospray ionization-mass spectrometry (SESI-MS) [10,11] or selected ion flow tube-mass spectrometry (SIFT-MS) [12,13] with the later one being able to directly quantify the concentration of target substance based on a gas phase ion-molecule reaction kinetics. Nevertheless, the protocols most commonly employed for the untargeted analysis of a wide range of VOCs typically utilize gas chromatography coupled with mass spectrometry (GC-MS). Notably, GC-MS requires the use of sample-preparation techniques. On one hand, this requirement is an obvious drawback compared to the convenience of direct analysis; on the other hand, however, the addition of a sample-preparation step enables the analysis of trace levels of diverse metabolites in complex matrices. Amongst these sample-preparation approaches, solid-phase microextraction (SPME) and sorbent tubes (sometimes referred to as solid-phase extraction (SPE) or simply thermal desorption (TD)) are the most common (see Tables 1 [10–12,14–58], 2 [40,59–63], and 3 [2,4–8,10,13,47,64–81]), although other protocols have been growing in popularity, including needle-trap microextraction (NTME) and thin-film microextraction (TFME).

The previous works on volatile bacterial metabolites, like the review by Bos et al. [82] which discussed specific compounds related to the six most abundant pathogens in sepsis, need to be updated as the substantial progress in this field has been done over the last decade of intensive research, particularly in the field of animal and human studies. In this paper, we present a comprehensive overview of the current state of the art and future perspectives regarding the use of microextraction techniques in the metabolomic analysis of pathogenic bacteria. In the first part of this paper, we review the analytical techniques that have been used for the direct analysis of bacterial VOCs with the potential for point-of-care applications and methods for the off-line analysis along with the various sample-preparation techniques required. In the subsequent sections, we discuss the *in vitro* and *in vivo* studies aimed at profiling the pathogen volatilome, including the newest achievements in animal models and clinical studies focused on diagnosis of bacterial infections in humans (Tables 2 and 3).

2. Methodological considerations in bacterial VOC analysis

2.1. On-line analysis of volatile metabolites

2.1.1. Sensor arrays

On-line analysis aims to detect patterns in chemical information (i.e., instrument response to a set of analytes), which can be effective in disease identification. The most common examples of on-line approaches are direct mass spectrometric techniques and sensor arrays. Sensor arrays are referred to as an electronic nose (E-Nose) because they electronically mimic the mammalian olfactory system via numerous sensors with different polarities, which are arranged in an electric circuit. When a gaseous sample passes a sensor array, the individual VOCs adsorb to the different sensors to varying degrees; this changes the mass or resistance of each sensor, thus altering the analog signal. The changes caused by exposure to particular VOCs are manifested as alterations in the output, which are recognized as a specific pattern by software utilizing supervised learning methods. This in turn enables a database of overall odor fingerprints (instead of a single substance) for training samples (diseased individuals), which can later be used to differentiate these VOC patterns from samples obtained from non-diseased individuals. As a result, E-Nose cannot be used to identify or screening unknown substances. Furthermore, the sensitivity and accuracy of E-Noses depend on the presence of high humidity and temperature, which requires them to be periodically calibrated against a set of VOCs stored in their database. On the other hand, E-Noses do not require sample preparation, are simple to use, and provide rapid results. Therefore, these devices are very well suited for point-of-care applications, and the literature contains several pilot studies that have demonstrated their usefulness in the detection of numerous diseases, including ventilator-associated pneumonia [83], bloodstream infection [84], and tuberculosis (TB) [5,6].

2.1.2. Direct spectrometric techniques

Direct mass spectrometric methods are suitable for the quantitative analysis of target compounds in gaseous matrices, even at trace levels (sub-ppb), without sample preparation. The most prominent methods in this class are: ion mobility spectrometry (IMS), SIFT-MS, proton transfer reaction mass spectrometry (PTR-MS), and SESI-MS.

There are several subtypes of IMS technique depending on ionization mode, applied electric field or gas dynamics, amongst which drift tube IMS, traveling wave IMS, trapped IMS, field asymmetric IMS and differential mobility are the most popular and commercially available. In the classic form, the analyte molecules are ionized at atmospheric pressure by high-energy electrons generated from a ^{63}Ni source (shielded in a chamber). The created ions move further through a drift tube in an inert gas and are separated under the influence of an electric field. Within the drift tube, an inert buffer gas has no directional flow and the relatively weak (tens of V/cm) electric field is applied uniformly in this separation space. At the specific electric field strength, small ions have higher mobility hence reach higher corresponding velocity than larger, less mobile ions which move slower hence reach the detector (Faraday plate) later. Other reviews also describes more detail technical information on the IMS technique [85]. The short analysis time (typically few minutes) and portability of the IMS instrument makes this technique very attractive for a rapid screening for possible infections on the basis of bacterial VOCs analysis. The promising results were achieved by Steppert et al. [86] who used IMS coupled to multicapillary column to analyze volatile compounds in headspace (HS) gas from seven different bacteria cultures, revealing that multi-capillary column IMS (MCC-IMS) is able to differentiate tested pathogens. Similarly, Kunze-Szikszay et al. [87] used the MCC-IMS to analyze bacterial VOCs and differentiate between 11 pathogenic species related to hospital-acquired infections.

Although SIFT-MS and PTR-MS are not handheld, they can be considered portable, which allows their use in point-of-care applications. In contrast, the SESI device is a specialized ion source that is an add-on to a high-resolution mass spectrometer, which is a very expensive apparatus immobilized in a laboratory and requires well-trained personnel for system operation and data interpretation. The uniqueness of each of the aforementioned direct-MS methods lies in their approach to ionizing the analyzed molecules. For instance, in SIFT-MS, microwave plasma is used to ionize air and water vapor to form three positive precursor ions, H_3O^+, $O_2^{+\bullet}$, and NO^+, as well as five negative precursor ions (O^-, OH^-, O_2^-, NO_2^-, and NO_3^-). These precursor ions are then separated into single ionic species using a quadrupole and injected into a carrier gas (helium or nitrogen), which is introduced to the flow tube where the ions interact with the VOCs constituting the sample. SIFT-MS's ability to provide very soft chemical ionization (resulting in simplified spectra) and well-defined gas phase reaction kinetics enables the simultaneous absolute quantification of concentrations of several volatile metabolites in real time without the need for external calibration with respective reference standards, which is

highly desirable in clinical settings. SIFT-MS has been applied successfully to analyze the volatile metabolites produced by pathogenic bacteria under *in vitro* conditions [88–91], as well as under *in vivo* conditions for the targeted analysis of hydrogen cyanide levels in exhaled breath samples from adult cystic fibrosis (CF) patients with and without *Pseudomonas aeruginosa* (*P. aeruginosa*) [71]. Furthermore, SIFT-MS has been applied for untargeted breath analysis to identify VOC-based classifiers that would enable the differentiation of children with CF and asthma from healthy children [92], as well as to determine pathogen-specific responses in a rat pneumonia model [60].

In the PTR-MS technique, similar reactant ions (precursors) are available to ionize the analytes' molecules. Amongst them, the proton transfer reaction stands for the basis of the PTR-MS technique. By definition, ionization with hydronium ions (H_3O^+) is possible only for VOCs with a proton affinity higher than that of a water molecule (166.5 kcal/mol). The water clusters of the protonated analytes are broken down in the collision dissociation chamber (CDC), which is situated directly after the drift tube; the resultant ions are then separated according to their mass-to-charge ratios (m/z) in the quadrupole and ultimately reach the detector. Similar to SIFT-MS, the PTR-MS does not require external calibration, as the concentration of the target analyte can be calculated based on the reaction constant and drift time, assuming no fragmentation of the protonated analyte and only one type of precursor ion (H_3O^+). However, ionized species often undergo fragmentation when the energy deposited in them becomes too high due to the increases in CDC voltage or the E/N ratio in the drift tube (where E stands for the electric field and N stands for the number of particles per cm^3). Additionally, it has been shown that sample humidity strongly affects fragmentation under PTR-MS conditions, such that lower water content in a sample leads to stronger fragmentation of the ionized analytes. This fragmentation not only hinders quantitative analysis (lowering the signal of $[M+H]^+$ and increasing the signal at other m/z), but it can also spoil the qualitative analysis. For instance, fragmentation can cause the hydroxyl group in ionized alcohol (C3 and larger) to dissociate immediately, thus precluding the detection of its protonated form. In such cases, the quantitation of a target analyte requires the application of a calibration factor for the most abundant ion (if a signal at certain m/z originates only from one compound), or signal recombination with a mathematical model (if different compounds contribute to the same m/z) [93].

Given these factors, the PTR-MS technique can be successfully applied for the analysis of volatile bacterial metabolites. For instance, Lechner et al. [58] used PTR-MS and VOC fingerprinting to differentiate *Helicobacter pylori*, *P. aeruginosa*, *Klebsiella*, *Citrobacter*, and *Staphylococcus aureus* (*S. aureus*) under *in vitro* conditions. Three years later, Bunge et al. [57] created a system capable of cultivating four bacteria cultures in parallel and employed HS analysis with PTR-MS in 36 min intervals to construct a fine time profile for the release of volatile metabolites from actively growing bacteria. The findings of their analysis revealed complex mass spectra ranging from 18 to 150 amu. Although only 10 VOCs were tentatively identified (methanol, acetaldehyde, ethanol, methanethiol, butanol, acetone, acetic acid, 2-butanone, methylated butanol, and indole), they were able to use all ions within the 40–150 amu range to successfully differentiate *E. coli*, *Shigella flexneri*, *Salmonella enterica*, and *Candida tropicalis* [57]. Similarly, O'Hara and Mayhew [56] used PTR-MS for discrete-time profiling of bacterial VOCs to investigate how different nutrients in culture media influence the emission of VOCs from *S. aureus* under *in vitro* conditions. Most recently, Roslund et al. [94] used PTR-time-of-flight (TOF)-MS for the on-line analysis of VOCs secreted from pathogenic oral bacteria *in vitro*.

The obvious limitation of SIFT-MS and PTR-MS techniques, namely the uncertain identification of some compounds due to the above-discussed reasons, is addressed in SESI, when coupled to the high-resolution mass spectrometer (such as an Orbitrap). There is evidence indicating that target volatile compounds are ionized in SESI via ion-molecule reactions (where charging ions originate from nanodroplets) rather than droplet-vapor charge exchange (i.e., analytes are not dissolved in droplets of electrospray solution and later reemitted, as initially thought) [95]. Since high-energy ions are involved neither in the creation of precursor ions nor in the ionization of analytes, SESI features remarkably low fragmentation and recorded spectra dominated by the bare ion peak. These characteristics enables SESI to provide better analyte identification (when coupled to high resolution-MS) compared to the SIFT- and PTR-based techniques. Furthermore, the sensitivity of SESI-MS analysis is correlated with the mass of volatile compounds, which makes this technique ideal for detecting low-volatility species in gas phase. This fills an important gap, as low-volatility compounds have been typically overlooked in other analytical techniques, even though their biological significance could be equal to, or even greater than, that of high-volatility low-molecular-weight metabolites.

SESI-MS has also been applied for VOC fingerprinting to differentiate *S. aureus*, *E. coli*, and *Streptococcus pneumoniae* in the HS of bacteria blood cultures [96,97], and to differentiate *E. coli*, *Salmonella Typhimurium*, and *S. aureus* in food modeling media [98]. In addition, SESI-MS has been applied in mouse models to differentiate selected pathogens [63,99] and to investigate the differences in volatile metabolites in susceptible and resistant strains of *S. aureus* [62]. Finally, this technique has also been applied in human studies for the real-time analysis of breath samples from adult [100] and pediatric [101] CF patients. Notably, the real-time breath analyses with SESI-MS were summarized and optimal conditions were recommended to standardize the analytical procedure and improve future inter-laboratory comparisons [102], but it still remains only semi-quantitative as discussed by Dryahina et al. [103].

2.2. Off-line analysis of volatile metabolites

The unambiguous identification of analytes is critical to all forms of basic research focusing on bacterial VOCs, regardless of whether the study in question aims to elucidate a metabolic pathway by characterizing factors affecting its activity, investigate inter-species interactions, or identify specific volatile biomarkers for use in the diagnosis of bacterial infections and monitoring of treatments. In most of these studies, untargeted analysis was employed in order to reveal the broadest possible range of bacterial metabolites. However, this type of analysis is very challenging in complex matrices, particularly in animal and human models, as, apart from the stable background, varying conditions related to immune activity in the host or the applied treatment can complicate the analysis and data interpretation. Therefore, such analysis requires the separation of sample constituents into single analytes and detection methods enabling the identification of each substance. In the case of VOCs, GC-MS is the gold standard, as it fulfills the aforementioned requirements. Selecting the appropriate chromatographic column and optimizing its working conditions (especially the temperature program) can enable the separation of over 100 compounds in a single run, while the application of two-dimensional GC (GC × GC) can increase this number to several hundred, and even thousands in particular cases. In the GC × GC technique, the eluate leaving the first column (which may still contain unresolved peaks) is trapped for a few seconds in a modulator (thermal, flow, or closed cycle loop),

where it is focused into a very narrow zone and re-injected into a second column for further chromatographic separation. The selection of both columns depends on the application and users' expertise. In VOC analysis, the primary column (1D) is typically a nonpolar porous layer open tubular (PLOT) column with an ID of 0.25 mm and a length of approximately 30 m, while the second column is a polar column with an ID of 0.1 mm and a length of approximately 1–2 m. Furthermore, the very narrow peak widths enabled by this method (down to 50 ms when a thermal modulator is used) necessitate the use of very fast scanning detectors. To this end, TOF mass spectrometer is often the most suitable option, as it is able to capture multiple wide-range mass spectra within a very short time-periods (equal to refocusing cycles in a modulator). In addition to providing higher resolving power, this feature substantially increases the sensitivity of GC × GC-MS compared to a one-dimensional setup. Nevertheless, GC × GC-MS systems are significantly more expensive than their 1D counterparts and require a well-trained specialist to adequately optimize the entire protocol and interpret the resultant data, which is not the case with standard GC-MS systems.

2.3. Sample-preparation techniques applied in chromatographic analysis of microbial VOCs

Since biological samples are very complex matrices, it is often necessary to add a sample-preparation step, which can become a bottleneck in the analytical protocol on one hand, but ensures isolation of the wide range of VOCs for precise and sensitive untargeted analysis on the other hand. Among the approaches available at present, microextraction techniques have been garnering increasing interest due to their lack of a need for organic solvents (which significantly reduces the cost and duration of the protocol) and non-destructive functionality, which allows samples to be stored as a biobank for use in further quality control or legacy metabolomic studies after extractions have been completed. The most suitable extraction techniques for offline GC-MS analysis are those that utilize adsorption process followed by thermal desorption. Apart from routine extraction parameters (e.g., exposure time, temperature, and sample volume), the type of stationary phase, its dimensions, and even the shape of the device can influence analyte recovery. Currently, the most commonly used devices for the analysis of VOCs in gaseous samples include SPME fibers (multibed-), sorption tubes, needle traps, and carbon meshes coated with a desired sorbent for TFME. Due to their exhaustive nature, sorption tubes and needle traps ensure lower detection limits (limits of detection (LODs) down to ppt_v) compared to equilibrium techniques (SPME and TFME) where extraction is performed from limited sample volume resulting in LODs typically at low-ppb_v levels. However, one should keep in mind that overall sensitivity of analysis depends not only on fine optimization of condition for sample preconcentration but also on GC and particularly MS settings (selected ion monitoring (SIM) mode instead of full scan, longer dwell time for quadrupole or elevated electron multiplier voltage, amongst others).

2.3.1. Sorption tubes

The first technique applied for adsorptive sample preconcentration (and VOC isolation) employed sorption tubes. It is an exhaustive technique, widely used to this day, due to its many advantages, including its ability to provide very low detection limits (typically at the ppt_v level) when a large volume of a gaseous sample (typically 0.1–1 dm^3) passes through the tube filled with a relatively large amount of adsorbent (100–500 mg). Apart from commercial sourcing, sorption tubes can be easily prepared in-house using adsorbents most suitable for the application at hand. Another valuable feature of sorbent tubes is their excellent ability to preserve the stability of the adsorbed sample, which enables its long-term storage (e.g., for shipping samples obtained in different locations to one analytical laboratory). Indeed, Ahmed et al. [104] observed no significant variations ($P = 0.514$; Kruskall-Wallis test) in the stability of breath samples collected via sorbent tubes after storage for up to 39 days at lowered temperatures (2–8 °C). Since strong adsorbents (e.g., carbon molecular sieves) are required to efficiently trap very volatile compounds, water uptake can be a limiting factor, as it can result in numerous undesired effects, such as the saturation of the adsorbent (loss of sensitivity), deterioration in refocusing (icing of a cold trap), and chromatographic separation (shifts in retention times hindering analyte identification). There are several ways to minimize excessive water uptake at different steps of extraction: 1) prior to adsorption by the application of drying agents, such as $CaCl_2$, $MgSO_4$, Na_2CO_3, K_2CO_3, $CaCO_3$, or permeation through Nafion membranes; 2) during adsorption by decreasing the sample's relative humidity by diluting it with dry gas or elevating the adsorption temperature; and 3) after adsorption via dry purging with purified gas prior to thermal desorption. Each of the aforementioned methods has advantages and drawbacks that should be considered when determining their suitability for a particular application. For instance, decreasing the sample's relative humidity during extraction, particularly via dilution with purified dry air, has been successfully applied in dynamic HS sampling from bacteria cultured *in vitro* [15]. Along with the superior analytical achievements of this technique, its costs are higher than related methods, as it requires the external apparatus for thermal desorption coupled to the GC-MS.

2.3.2. SPME

SPME, which is based on the use of nitinol fibers coated with μm-layer adsorbent, is a technique that both eliminates the water-uptake problem associated with sorbent tubes and enables the automation of the sample extraction and desorption steps due to its compatibility with GC equipment (utilizing, inter alia, such autosamplers as CombiPAL from CTC Analytics, MPS from Gerstel, Concept96 from PAS Technology). Since its introduction in the early 1990s by Janusz Pawliszyn and his colleagues, SPME has become the most widely used and versatile sampling technique for the analysis of volatile metabolites in clinical, pharmaceutical, and biotechnological research [105]. For instance, SPME has been successfully applied for breath analysis to detect pneumonia in animal models. In this work, SPME fibers were placed inside the endotracheal tube of rabbits infected with *E. coli, S. aureus*, or *P. aeruginosa* to extract specie-specific VOCs [40]. SPME has also been successfully applied for the analysis of bacterial metabolites in diverse biological samples, such as cerebrospinal fluid [64] and sputum from CF patients [73], and blood, sweat, urine, feces, tissues, exhaled breath gas, cell lines, and bacteria cultures [105]. Although the low stability of adsorbed VOCs precludes the long-term storage and transportation of loaded SPME fibers, its excellent compatibility with GC-MS equipment and resulting high degree of automation allows the entire protocol to be executed on one system. As demonstrated by Timm et al. [37], robotic autosamplers can be used to synchronize bacteria cultivation under well-controlled conditions (temperature and stirring) with HS extraction at pre-programmed time intervals, followed by thermal desorption in an GC injector for final analysis. This strategy was

also applied by Azzollini et al. [106] to investigate the dynamics of metabolite induction at four different time points (2, 4, 7, and 9 days) in fungal mono- and co-cultures of *Eutypa lata* and *Botryospearia obtuse*. Their findings revealed that the production of particular metabolites (e.g., 2-nonanone) was induced only in the co-cultures and did not occur at all in the mono-cultures throughout the duration of the study. Moreover, Azzollini et al. [106] further demonstrated the antifungal properties of 2-nonanone, as their results clearly indicated that microorganisms triggered a chemical defense in response to the presence of other microorganisms. Researchers continue to develop and refine SPME approaches, both with respect to the employed derivatization strategies [107] and the novel stationary phases used in the fiber coatings, such as graphene oxide [108] or its modification with polyaniline/polypyrrole [109] or polypyrrole-enhanced titania nanoparticles [110], to improve the method's sensitivity, reproducibility, and linearity range.

2.3.3. NTME

Introduced in 2001 by Koziel et al. [111], needle-trap devices (NTD) consist of a steel needle packed with adsorbent and combine the advantages of equilibrium-based microextraction offered by fibers with the exhaustive nature of sorption tubes. Due to its characteristic geometry and amount of adsorbent, NTME is able to address many of the concerns associated with SPME, such as diffusion-related limitations, partition-coefficient limitations, adsorption capacity, fragility, and limitations related to long-term storage (up to few days). Altogether, NTME offers considerable benefits over SPME, including the quantitative adsorption of analytes with smaller values of log*P*, reduced extraction times, and improved sensitivity and stability during sample storage. However, it is important to consider a number of factors when optimizing the NTME sampling procedure, inter alia: the employed sampling mode (bi-directional vs. continuous flow); the composition of the NTD (the type of adsorbent and number of sorbent beds); the profile of the NTD (a tip-hole vs. a side-hole profile); or the selected sampling temperature and flow rate. Despite their similarities and exhaustive nature, needle traps and sorption tubes are affected by certain factors very differently. For instance, whereas water uptake is always highly undesirable in sorption tubes, it improves the desorption efficiency in needle traps [112] by creating an expansive flow (rapid vaporization of the adsorbed water) that facilitates the transfer of the desorbed VOCs from the NTD towards the GC column. This eliminates the need for an external equipment for thermal desorption (required for sorption tubes), and ensures that the entire extraction process (sampling-injection cycle) can be easily automated in specific applications (similarly to SPME), significantly reducing time and cost of the analysis.

A highly interesting structural modification to an NTD was recently introduced by Zeinali and Pawliszyn [113], who implemented a sorbent consisting of a light porous fibrous structure made of an aerogel based on polyacrylonitrile to enable the adsorption of VOCs bound to aerosol particles in humid samples. This modification proved to be very effective, as it allowed exhaled breath gas and condensate to be extracted by a single device, thus facilitating the simultaneous analysis of volatile and droplet-bound, particularly polar, compounds [114]. Another direction in the development of NTME is the search for new extraction phases. In recent years, sorbents such as graphene/polyaniline [115], molecular-imprinted polymers [116], and metal-organic frameworks (MOFs) (e.g., zirconium-based MOF [117] or titanium-based MOF [118]) have garnered increasing attention, as they all enable superior sensitivity (LODs lower than ng/L) and stability (minimal or no sample loss after weeks of storage). However, none of these coatings have been applied for the analysis of microbial VOCs thus far.

2.3.4. TFME

TFME, which was introduced in 2003 by Bruheim et al. [119] is a modified form of SPME that provides enhanced extraction efficiency and shorter sampling times due to the use of devices with increased surface area, and therefore, larger stationary phase surface areas, instead of increasing the volume of stationary phase, which prolongs equilibration time, hinders desorption efficiency, increases the background, and bleed from extraction phase itself. In 2015, Grandy et al. [120] proposed a new generation of TFME that further increased the area-to-volume ratio and reduced the bleeding of siloxanes by depositing an extraction phase onto a carbon (instead of fiberglass) mesh using high-density polydimethylsiloxane (PDMS) prepolymer as a thermally stable glue. Recently, Emmons et al. [121] published a review of the diverse coating methods currently in use for the preparation of TFME devices. TFME sheets are prepared using most of the same stationary phases as SPME devices, including divinylbenzene (DVB), carboxen (CAR), and PDMS. However, the highly electronegative elements comprising the polar group of an analyte weaken its $\pi-\pi$ interactions with the sorbent. This can be counteracted by coating the carbon mesh with a stationary phase composed of hydrophilic-lipophilic balanced (HLB) particles, as these particles, which are synthesized by incorporating *N*-vinyl-pyrrolidone groups into a DVB polymer, enable enhanced adsorption efficiency for oxygenated VOCs (alcohols, ketones, and aldehydes). In a recent study, the application of TFME with HLB-based coatings for the trace analysis of volatile metabolites released from A549 lung cancer cells enabled single- and sub-ppb detection limits for polar VOCs [122]. Since TFME meshes have only recently been upgraded to provide highly reproducible and sensitive sample preparation for GC-MS, their application for the analysis of volatile metabolites has been very limited [114,122]. Nonetheless, given the many benefits of TFME discussed in this section, its further application in *in vitro* and *in vivo* studies is expected.

3. *In vitro* studies

Continuous progress in the analysis of microbial VOCs, particularly the discovery and evaluation of bacterial biomarkers ("fingerprints"), requires successive *in vitro* and clinical *in vivo* studies. Although markers detected *in vitro* may not always be directly transferrable to *in vivo* conditions, models based on the *in vitro* analysis of the microbial origins of VOCs are required before introducing VOC analysis into clinical practice. The most frequently investigated and best known bacterial species include *E. coli*, *S. aureus*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), and *P. aeruginosa*, which along with *Enterococcus faecium* form the so-called ESKAPE group [123]. These bacteria are of particular importance due to their high rates of multidrug resistance and their dominant role in the development of hospital acquired infections (HAI), including VAP, sepsis, urinary tract infections (UTIs), and wound infections. *Mycobacterium* spp. is another notable threatening pathogen, as TB can remain latent for a long period of time. In this section, we review *in vitro* studies conducted over the past decade that directly address (in a single experiment) pathogen fingerprinting and factors influencing the production of VOCs. An overview of *in vitro* studies on bacterial VOCs including analyzed material, analytical platform used, aims and observations is given in Table 1.

Table 1
An overview of *in vitro* studies on bacterial volatile organic compounds.

Material	Analytical platform	Aims	Observations	Refs.
E. coli (CTX-M-type ESBL producing isolate and non-ESBL producing reference strain) grown in LB broth.	SPME-GC-MS/MOS	• Discriminate between ESBL and non-ESBL *E. coli* strains. • Determine how cephalosporines (cefotaxime and cefepime) affect the VOC profiles of ESBL and non-ESBL *E. coli*. • Compare MOS and MS detection.	• 4 discriminative compounds for comparison of ESBL vs. non-ESBL *E. coli*. • 5 discriminative compounds for comparison of the addition of CTX vs. CFP. • 50 features detected with both MOS and MS; 24 additional features for MOS; and 11 additional features for MS.	[14]
S. aureus (JE2 and Cowan1)[a] grown on fresh blood agar.	SESI-HRMS	• Assess the feasibility of SESI-HRMS as a tool for the rapid detection and differentiation of bacterial infections.	• 51 *S. aureus*-specific features (JE2 and Cowan1). • 302 features unique to *S. aureus* JE2. • 15 features unique to *S. aureus* Cowan1. • Real-time detection of unique features of *S. aureus* within minutes of growth.	[10]
Carbapenem susceptible (DSMZ 681) and resistant (DSMZ 103517) *K. pneumoniae* and clinical isolates grown in TSB.	TD-GC-MS (sorbent tubes)	• Characterize the kinetics of the release and uptake of VOCs. • Compare VOC profiles of reference and clinical strains.	• 44 significant VOCs for susceptible *K. pneumoniae* (19↑/13^{tm}/12↓). • 30 significant VOCs for resistant *K. pneumoniae* (14↑/11^{tm}/5↓). • 33 of 44 VOCs for clinical isolates confirmed in the *in vitro* model.	[15]
S. aureus (DSM2569 and DSM799), *P. aeruginosa* (DSM19880 and DSM25642), and *E. coli* (DSM30083 and DSM105372) grown in BHI, TSB, or LB.	SPME-GC-MS	• Analyze VOC profiles of *S. aureus*, *P. aeruginosa*, and *E. coli* and in three different growth media (BHI, LB, and TSB). • Investigate strain-level specificity.	• For *S. aureus*: 11 VOCs in BHI; 13 VOCs in TSB; and 11 VOCs in LB. • For *P. aeruginosa*: 22 VOCs in BHI; 23 VOCs in TSB; and 28 VOCs in LB. • For *E. coli*: 41 VOCs in BHI; 25 VOCs in TSB; and 37 VOCs in LB. • Strain-level variation in *P. aeruginosa* and *E. coli*.	[16]
MTB complex, *Mycobacterium avium* complex, and *Mycobacterium abscessus* complex grown in 7H9 broth.	SPME-GC-qMS	• Analyze culture HSs to identify discriminative VOCs for each species.	• 13 discriminative features for all types of mycobacteria.	[17]
S. aureus[a] (ATCC 12600) in BHI, MHB, LB, and TSB.	SPME-GC × GC-TOF-MS	• Determine how the culture medium influences the bacterial VOC profile.	• 31 VOCs in LB only; 24 VOCs in MHB only; 19 VOCs in BHI only; and 39 VOCs in TSB only.	[18]
E. coli (CTX-M-type ESBL producing isolate and non-ESBL producing reference strain) grown in LB broth.	SPME-GC-MS	• Discriminate between susceptible and resistant strains.	• 5 VOCs associated with resistant *E. coli*. • 2 VOCs associated with susceptible *E. coli*.	[19]
P. aeruginosa DSM 50071 (ATCC 10145) grown on LB agar and LB with KNO_3 agar.	TFME-GC-MS	• Develop a detailed method for the analysis of VOCs in *in vitro P. aeruginosa* biofilms in aerobic/anaerobic conditions.	• 11 identified VOCs released by *P. aeruginosa* (8 for aerobic conditions and 3 for anaerobic conditions).	[20]
S. aureus (DSM2569 and DSM799), *P. aeruginosa* (DSM19880 and DSM25642), and *E. coli* (DSM30083 and DSM105372)[a] grown in TSB.	SPME-GC-MS	• Analyze VOC profiles of *S. aureus*, *P. aeruginosa*, and *E. coli* in infected wounds. • Investigate species- and strain-level diversity. • Conduct a correlation study of VOC profiles and cell growth in *S. aureus* and *P. aeruginosa*.	• 34 VOCs for *E. coli*; 22 VOCs for *P. aeruginosa*; and 26 VOCs for *S. aureus*. • Strain-level variation in *P. aeruginosa* and *E. coli*. • Aldehydes ↓ following the incubation of *S. aureus* and *P. aeruginosa*. • Alcohols ↑ in 8−24 h; alcohols↓, acids↑ in 32−48 h of growth *S. aureus*.	[21]
S. aureus[a] (ATCC 12600) grown in BHI, LB broth, MHB, and TSB.	SPME-GC × GC-TOF-MS	• Determine the influence of culture medium on bacterial VOC profile.	• 36 VOCs released by *S. aureus*. • For media containing free glucose (BHI and TSB), VOCs profile is dominated by acids and esters (67%). • For low-glucose media (LB and MHB), VOC profile is dominated by ketones.	[22]
P. aeruginosa (clinical isolates) grown in LB broth.	GC × GC−TOF-MS	• Analyze changes in the volatilome of CF-associated *P. aeruginosa* isolates.	• 26 VOCs not previously reported for *P. aeruginosa*.	[23]
Human cystic fibrosis bronchial epithelial cells infected with *P. aeruginosa* (PAO1), RSV, or both.	SPME-GC × GC-TOF-MS	• Discriminate between CFBE infected with *P. aeruginosa*, RSV, or co-infection based on VOC analysis.	• 34 discriminative VOCs for *P. aeruginosa* infection vs. control. • 16 discriminative VOCs for RSV-*P. aeruginosa* co-infection vs. control. • 6 discriminative VOCs for *P. aeruginosa* infection vs. RSV infection. • Volatile signature of co-infected samples (*P. aeruginosa*+/RSV+) dominated by the signature produced by *P. aeruginosa*.	[24]
E. coli (clinical isolates) grown in blood.	SPME-GC-MS CDI-MS CID	• Analyze indole profiles during culture growth.	• Indole production was substantially increased during the early culture time of bacterial growth (6−7 h).	[25]
P. aeruginosa, Klebsiella spp., *E. coli, S. aureus,* and *Acinetobacter* spp.[a] (clinical urine or blood isolates) grown in MHB.	SPME-GC × GC-TOF-MS	• Analyze VOC profiles to differentiate pathogens.	• 33 discriminatory volatiles (15 abundant for *P. aeruginosa*; 20 abundant for *E. coli*; 14 abundant for *S. aureus*; 13 abundant for *Acinetobacter* spp.; and 18 abundant for *Klebsiella* spp.).	[26]

(*continued on next page*)

Table 1 (*continued*)

Material	Analytical platform	Aims	Observations	Refs.
E. coli, K. pneumoniae, and *P. aeruginosa* (clinical isolates) grown in NB.	TD-GC-MS	• Analyze VOC profiles. • Differentiate CPN and CPFX susceptible and resistant strains.	• 31 discriminative VOCs between *S. aureus*, *P. aeruginosa*, and *K. pneumoniae.* • 9 discriminative VOCs for the comparison of CPN-susceptible vs. CPN-resistant without the addition of CPN (16 with addition). • 22 discriminative VOCs for the comparison of CPFX-susceptible and CPFX-resistant without the addition of CPFX (5 with addition).	[27]
E. coli (DSM 25944), *S. aureus* (DSM 13661), and *P. aeruginosa* (DSM 1117) in human whole blood.	GC-IMS	• Analyze VOC profiles for the early recognition and differentiation of BSI-related pathogens.	• For *E. coli*: 1 VOC ↓ and 34 VOCs ↑. • For *S. aureus*: 18 VOCs ↑. • For *P. aeruginosa*: 2 VOCs ↓ and 3 VOCs ↑.	[28]
K. pneumoniae (clinical blood isolates) grown in BHI, LB broth, MHB, or TSB.	SPME-GC × GC-TOF-MS	• Assess the core and media-specific components of the *K. pneumoniae* volatile metabolome.	• 365 VOCs overall. • 36 core VOCs (released by all isolates). • 329 media-dependent VOCs	[29]
KPC, non-KPC *K. pneumoniae*,CPE, and non-CPE *E. coli*[a] isolates grown in MHB.	SPME-GC × GC-TOF-MS	• Differentiate between KPC and non-KPC strains.	• For KPC/CPE: 17 core VOCs and 16 pan VOCs. • For non-KPC/CPE: 26 core VOCs and 8 pan VOCs.	[30]
MSSA (ATCC 29213) and *P. aeruginosa*[a] (ATCC 27316) biofilms on 13 mm diameter Nunc™ Thermanox Coverslips.	TD-GC-MS	• Identify the biofilms' unique VOC profiles.	• 4 VOCs specific to *S. aureus.* • 8 VOCs specific to *P. aeruginosa.*	[31]
MSSA *S. aureus* (ATCC 29213) and *P. aeruginosa* (ATCC 27316) biofilms on 13 mm diameter Nunc™ Thermanox Coverslips.	TD-GC-MS	• Assess the usefullness of VOCs in monitoring response to CPFX treatment. • Measure anti-biofilm efficacy of electrical stimulation.	• 5 VOCs unique to *P. aeruginosa* biofilms. • 2 VOCs specific for CPFX-treated MSSA biofilms. • 2 VOCs significantly reduced in CFPX-treated MSSA biofilms. • 6 VOCs significantly reduced in CFPX-treated *P. aeruginosa* biofilms. • 4 VOCs significantly reduced in CFPX and electrical stimulation-treated *P. aeruginosa* biofilms.	[32]
Lung carcinoma A549 epithelial cell line (ATCC CCL 185) infected and non-infected with *P. aeruginosa* (ATCC 10145).	TD-GC-MS (sorbent tubes)	• Discriminate between A549 infected and not infected with *PA* and treated with hydrogen peroxide (oxydative stress).	• 1000 significantly different features between infected vs. uninfected A549. • 800 significantly different features between treated vs. untreated (Mann-Whitney U test).	[33]
Enterobacter cloacae (DSM 30054) and *P. aeruginosa* (ATCC 10145) mono- and co-cultures grown in ASM.	TD-GC-MS (sorbent tubes)	• Analyze VOC profiles and discriminate between *Enterobacter cloacae* and *P. aeruginosa* mono- and co-cultures.	• For *Enterobacter cloacae*: 10 VOCs↓and 8 VOCs↑. • For *P. aeruginosa*: 13 VOCs ↓ and 4 VOCs ↑. • For co-culture: 10 VOCs ↓ and 6 VOCs ↑.	[34]
MSSA (RN450) and MRSA (450 M) grown in LB broth.	SESI-MS/MS	• Discriminate between MSSA and MRSA.	• Significant differences in 3 features ($P < 0.01$) between MSSA and MRSA.	[11]
E. coli (ATCC 25922) grown on TSA plates and in TSB.	SBSE-GC-MS	• Compare two types of SBSE (PDMS and EG-S).	• 93 VOCs for PDMS bar. • 40 VOCs for EG-S bar.	[35]
Mycobacterium avium spp. *paratuberculosis* (DSMZ 441333), *Mycobacterium bovis* (DSMZ 43990), *Mycobacterium intracellulare* (DSMZ 43223), and *Mycobacterium avium* spp. *avium* (DSMZ 44156)[a] grown on HEYM-MJ.	NT-GC-MS	• Determine unique VOC profiles and a set of substances indicating the presence of mycobacteria in general.	• For *Mycobacterium avium* spp. *paratuberculosis*: 6 VOCs ↓ and 16 VOCs ↑. • For *Mycobacterium bovis*: 9 VOCs ↓ and 15 VOCs ↑. • For *Mycobacterium intracellulare*: 7 VOCs ↓ and 22 VOCs ↑. • For *Mycobacterium avium* spp. *avium*: 10 VOCs ↓ and 7 VOCs ↑.	[36]
P. aeruginosa[a] (ATCC 47085) grown in MOPS glucose, MOPS glucose + EZ, and TSB.	SPME-GC-MS	• Characterize bacterial VOC profiles in various culture conditions.	• 17 VOCs released by *P. aeruginosa*: 7 in MOPS glucose; 9 in MOPS glucose + EZ; and 15 in TSB. • *P. aeruginosa* produces longer-chain VOCs in higher amounts at 37 °C vs. 30 °C.	[37]
E. coli (ATCC 25922) and *S. aureus*[a] (ATCC 25923) grown in MHB and TSB.	SPME-GC-MS	• Identify VOC profiles.	• 16 VOCs specific to *E. coli.* • 37 VOCs specific to *S. aureus.*	[38]
P. aeruginosa (clinical isolates: eye, ear, respiratory tract, abdomen, urinary tract, blood, and skin) grown in LB broth.	SPME-GC × GC-TOF-MS	• Comparative analysis of the volatile metabolomes of *P. aeruginosa* clinical isolates.	• 391 VOCs overall. • 70 core VOCs (released by all isolates).	[39]
E. coli (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 25923) grown in paracancerous human lung tissue.	SPME-GC-MS	• Identify characteristic VOCs. • Assess *in vitro* model to identify the most common pneumonia causative pathogens.	• 6 VOCs for *S.aureus* • 4 VOCs for *E. coli.* • 5 VOCs for *P. aeruginosa.*	[40]
P. aeruginosa (ATCC 27853), *A. baumannii* (ATCC 19606), and *K. pneumoniae* (ATCC 700683) grown in MHB and TSB.	SPME-GC-MS	• Identify characteristic VOCs.	• 10 VOCs specific to *P. aeruginosa.* • 8 VOC specific to *A. baumannii.* • 24 VOCs specific to *K. pneumoniae.*	[41]

(*continued on next page*)

Table 1 (*continued*)

Material	Analytical platform	Aims	Observations	Refs.
Mycobacterium avium ssp. *Paratuberculosis* (04A0386 and 05A2431) grown in HEYM-MJ.	NTME-GC-MS and SPME-GC-MS	• Compare SPME and NTME analysis of complex VOC patterns.	• 72 VOCs overall. • 18 VOCs exclusively detected by SPME.	[42]
Mycobacterium avium, Mycobacterium intracelulare, Mycobacterium xenopi, and *Mycobacterium bovis* (clinical blood, sputum, abscess, or pleural fluid isolates, 2 vaccine strains) grown in 7H9 broth.	SPME-GC × GC-TOF-MS	• Analyze VOCs produced by *Mycobacterium* species.	• 6 VOCS for *Mycobacterium avium.* • 10 VOCs for *Mycobacterium bovis.* • 6 VOCs for *Mycobacterium intracellulare.* • 3 VOCs for *Mycobacterium xenopi.*	[43]
S. aureus (ATCC 6538) and *E. coli*[a] O157:H7 (CICC 21530) mono- and co-cultures grown in TSB.	SPME-GC-MS	• Characterize bacterial VOC profiles in mono- and co-cultures.	• 7 VOCs specific to *E. coli.* • 3 VOCs specific to *S. aureus.* • *S. aureus* growth was supressed by *E.coli* in co-culture.	[44]
P. aeruginosa[a] grown in LB broth.	SPME-GC × GC-TOF-MS	• Discriminate CF-associated bacteria based on VOC profiles. • Analyze the influence of storage conditions.	• 38 discriminative VOCs for causative pathogen differentiation. • 25 discriminative VOCs for the comparison of short-term vs. long-term storage.	[45]
K. pneumoniae (ATCC 13883) grown in TSB and human blood.	SPME-GC × GC-TOF-MS	• Analyze VOC profiles of *K. pneumoniae* in human blood.	• 33 VOCs specific to *K. pneumoniae* blood culture vs. sterile blood.	[46]
P. aeruginosa and *S. aureus*[a] (clinical BAL/sputum and blood isolates) grown in BHI, MHB, and NB.	SIFT-MS	• Analyze VOCs emitted by certain bacterial species grown in various media.	• 4 VOCs specific to *P. aeruginosa.* • 1 VOC specific to *S. aureus.*	[12]
Lung carcinoma A549 epithelial cell line cells infected with *S. aureus* (DSM 20231) and *P. aeruginosa* (PaO1)[a].	SPME-GC-MS	• Develop a method for rapid breath analysis in CF patients.	• 1 VOC specific to *P. aeruginosa.* • No VOCs specific to *S. aureus.*	[47]
E. coli (NCTC 10418), *K. pneumoniae* (NCTC 9528), and *S. aureus* (NCTC 6571) grown in TSB, BHI broth, or EF broth.	SPME-GC-MS	• Characterize bacterial VOC profiles for various culture conditions and collection methods	• For *E. coli*: 6 VOCs in BHI; 7 VOCs in TSB; and 5 VOCs in EF. • For *K. pneumoniae*: 4 VOCs in BHI; 4 VOCs in TSB; and 4 VOCs in EF. • For *S. aureus*: 4 VOCs in BHI; 4 VOCs in TSB; and 4 VOCs in EF.	[48]
E. coli (ATCC 25922 and 35218), *P. aeruginosa* (ATCC 27853), *S. aureus* (MSSA and MRSA clinical isolates), and *K. pneumoniae* (ATCC 700683) grown in MHB.	TD-GC-TOF-MS (sorbent tubes)	• Analyze VOC profiles.	• For *E. coli*: 6 VOCs ↓ and 7 VOCs ↑. • For *P. aeruginosa*: 6 VOCs ↓ and 5 VOCs ↑. • For *S. aureus*: 6 VOCs ↓ and 5 VOCs ↑. • For *K. pneumoniae*: 6 VOCs ↓ and 6 VOCs ↑.	[49]
E. coli (ATCC 11775, DSM 30083) and *P. aeruginosa* (ATCC 10145, DSM 50071) grown in LB broth + tryptophane or glucose + tryptophane.	NT-GC-qMS	• Analyze growth-dependent VOC profiles.	• For *E. coli*: 5 VOCs ↓ and 4 VOCs ↑. • For *P. aeruginosa*: 7 VOCs ↓ and 4 VOCs ↑.	[50]
E. coli (DH10B) grown in whole blood or LB broth.	GC-FID-ECD-MSD	• Determine specific VOC profiles in whole human blood.	• 6 VOCs ↑ for *E. coli* in whole blood. • 10 VOCs ↑ for *E. coli* in LB.	[51]
E. coli (DSM 1103), *K. pneumoniae* (DSM 2026), *P. aeruginosa* (DSM 46358), and *S. aureus* (DSM 13661)[a] grown on Columbia sheep blood agar.	MCC-IMS TD-GC-MS (sorbent tubes)	• Demonstrate the usefullness of MCC-IMS VOC profiling to different human pathologic bacteria.	• For *E. coli*: 11 VOCs by IMS and 22 VOCs by GC-MS. • For *K. pneumoniae*: 2 VOCs by IMS and 4 VOCs by GC-MS. • For *P. aeruginosa*: 2 VOCs by IMS and 7 VOCs by GC-MS. • For *S. aureus*: 3 VOCs by IMS and 3 VOCs by GC-MS.	[52]
S. aureus (ATCC 25923) and *P. aeruginosa* (ATCC 27853) grown in TSB.	TD-GC-MS (sorbent tubes)	• Characterize the release and consumption of VOCs.	• For *S. aureus*: 1 VOC ↓ and 32 VOCs ↑. • For *P. aeruginosa*: 12 VOCs ↓ and 37 VOCs ↑.	[53]
MTB[a] grown on 7H11 solid medium or in 7H9 broth and Sauton liquid media.	CLSA-GC-EIMS	• Identify VOC profiles.	• 20 VOCs released on 7H11 solid medium. • 15 VOCs released in 7H9 broth.	[54]
P. aeruginosa (sputum and blood isolates) grown in MHB.	SPME-GC-MS SIFT-MS	• Compare SPME-GC-MS and SIFT-MS for the analysis of bacterial VOC profiles.	• 27 distinct VOCs for GC-MS analysis. • 28 distinct VOCs for SIFT-MS analysis.	[55]
S. aureus (clinical isolate) grown in NB, BHI, and DB.	PTR-MS	• Evaluate the usefullness of PTR-MS for volatilome analysis and identification depending on media type.	• In NB: *m*/*z* 61 (acetic acid) ↑ and other *m*/*z* [tm]. • In dextrose: *m*/*z* 55 [tm] and other *m*/*z* ↑. • In BHI: *m*/*z* 45 (acetaldehyde); *m*/*z* 49 (methanethiol); *m*/*z* 61 (acetic acid) [tm]; and other *m*/*z* ↑.	[56]
E. coli[a] (DSMZ 30083) grown in NB.	PTR-MS	• Evaluate the usefulness of PTR-MS in volatilome analysis. • Detail VOC profiling at short time-intervals. • Differentiate pathogens based on VOC profiles.	• 23 features for *E. coli.*	[57]

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Table 1 (*continued*)

Material	Analytical platform	Aims	Observations	Refs.
E. coli, *K. pneumoniae*, and *P. aeruginosa* grown on MacConkey agar and *S. aureus* (clinical isolates)[a] grown on mannitol-salt agar.	PTR-MS	• Determine unique VOC profiles.	• *K. pneumoniae* produces larger amounts of VOCs with low atomic masses.	[58]

[a] Authors also investigated other bacteria beyond the scope of this review; ↑: elevated; ↓: reduced; [tm]: temporary maximum. *E. coli*: *Escherichia coli*; ESBL: extended-spectrum beta-lactamase; LB: Luria-Bertani broth; SPME: solid-phase microextraction; GC-MS: gas chromatography-mass spectrometry; MOS: metal oxide sensor; VOCs: volatile organic compounds; CFP: cefepime; *S. aureus*: *Staphylococcus aureus*; SESI-HRMS: secondary electrospray ionization-high resolution mass spectrometry; *K. pneumoniae*: *Klebsiella pneumoniae*; TSB: trypticase soy broth; TD: thermal desorption; *P. aeruginosa*: *Pseudomonas aeruginosa*; BHI: brain-heart infusion broth; qMS: quadrupole mass spectrometry; MHB: Mueller-Hinton broth; GC × GC-TOF-MS: two-dimensional gas chromatography time-of-flight mass spectrometry; CF: cystic fibrosis; CFBE: cystic fibrosis bronchial epithelium; RSV: respiratory syncytial virus; CDI-MS: corona discharge ionization mass spectrometry; NB: nutrient broth; CPN: cephalexin; CPFX: ciprofloxacin; IMS: ion-mobility spectrometry; BSI: bloodstream infections; KPC: *Klebsiella* producing carbapenemase; CPE: carbapenemase-producing *Enterobacteriaceae*; MSSA: methicillin-susceptible *S. aureus;* ASM: artificial sputum medium; MRSA: methicillin-resistant *S. aureus*; SBSE: stir-bar sorptive extraction; TSA: tryptic soy agar; PDMS: polydimethylsiloxane; EG-S: ethylene-glicol silicone; HEYM-MJ: Herold's Egg Yolk Agar supplemented with amphotericin, nalidixic acid, vancomycin, and mycobactin J; NT: needle trap; MOPS: minimal medium; *A. baumannii*: *Acinetobacter baumannii*; NTME: needle-trap microextraction; BAL: bronchoalveolar lavage; SIFT-MS: selected ion flow tube mass spectrometry; EF broth: enteric fermentation base broth; FID: flame ionization detector; ECD: electron capture detectors; MSD: mass selective detector; MCC: multi-capillary column; CLSA: closed-loop stripping analysis; EIMS: electron impact mass spectrometry; DB: dextrose broth; PTR: proton-transfer reaction.

3.1. VOCs with potential significance as biomarkers of bacterial species

A large proportion of microbial VOC analyses have focused on identifying likely specific biomarkers of infections caused by certain bacteria species. However, to be classified as a biomarker, a compound must undergo the 5 phases outlined by the Early Detection Research Network (EDRN) [124] and meet the criteria for crucial parameters (i.e., sensitivity, specificity, robustness, accuracy, and reproducibility). Although catchy, the term, "biomarker," is overused in the study of bacterial infections; therefore, we suggest avoiding it, especially in initial basic research, such as *in vitro* studies. To provide a concise summary of the latest significant technological and biomedical advances in the analysis of bacterial VOCs, we focus only on bacteria belonging to the ESKAPE group and *Mycobacterium tuberculosis* (MTB). Since there are tens of *in vitro* studies that fulfill this inclusion criterion, consequently revealing the existence of more than a hundred compounds, we further narrow our index of volatile metabolites only to those reported in at least two unrelated experiments focusing on the same pathogen of interest. This filtering approach yielded a list of 93 metabolites, which are shown in Table S1 [2,12,14–21,23–29,31–39,41,43,44,46,48–52,54,55,57,72,125].

The vast majority of the volatile metabolites in Table S1 were present in relation to more than one bacteria species. In part, the identification of these VOCs for multiple pathogens was made possible through the use of highly sensitive analytical methods (both for sample preconcentration and final detection, as discussed in the previous sections) capable of detection at ultra-trace concentrations (ppt level). On the other hand, the metabolites listed in Table S1 are also indicative of the similarities in the metabolic pathways pivotal for bacteria cell survival, such as pyruvate metabolism via glycolytic fermentation and lactate converting fermentations. In particular, all the bacteria discussed in this section produce very volatile, low-molecular alcohols (e.g., ethanol, 2-methyl-1-propanol, 1-butanol, and 3-methyl-1-butanol) and acids (e.g. acetic acid and butanoic acid). However, there are also volatile compounds with metabolisms that substantially vary from one another, even at an intra-species level. While the reason for this variation remains unknown, it may potentially be related either to growth conditions, or to metabolic discrepancies at the strain level. For example, the findings of independent experiments found that aldehydes (e.g., 3-methylbutanal) were both produced (secreted) and consumed (taken up) *in vitro* by the same species of bacteria.

Regarding VOCs found in at least two independent studies on the pathogen of interest, *P. aeruginosa* generated the highest number of volatile metabolites from diverse chemical classes (n = 78), followed by *E. coli* (n = 64), *K. pneumoniae* (n = 42), and *S. aureus* (n = 40). Surprisingly, *A. baumannii* released very few compounds, with only 15 metabolites being reported in a total of four independent studies. Importantly, 14 of the 15 metabolites produced by *A. baumannii* were reported in a single *in vitro* study on this pathogen; as such, these metabolites are listed in Table S1 in relation to their detection in studies focusing on other bacteria. Since only 2,5-dimethylpyrazine was confirmed in two independent studies on *A. baumannii*, there remain no findings linking any specific VOCs to this specie.

Regarding the species-specific VOCs for the remaining pathogens, the findings of Tait et al.'s [48] multibacteria *in vitro* study indicated that indole is the characteristic VOC of *E. coli*. Similarly, Chen et al. [126] also observed the presence of indole in the HS of *E. coli* bacteria cultures. Indole is released as a result of the conversion of tryptophan by the enzyme, tryptophanase [35], and is responsible for the putrid odor characteristic to *E. coli* cultures [48,127]. As indole has also been detected in numerous other *in vitro* studies on *E. coli* [16,21,25,35,38,49,50,52], it can be concluded that it is the best known characteristic marker of the presence of *E. coli*. Although indole has been confirmed as a product of *E. coli* in as many as 13 independent *in vitro* experiments, findings have shown that it is not unique for *E. coli* as it is also released by *S. aureus* [38], *K. pneumoniae, A. baumannii* [41], and *P. aeruginosa* [41,55,72]. While *E. coli* is also characterized by the release of the ketones, 2-nonanone [16,44] and 2-heptanone [14,44], the emission of these compounds has also been found to be significant for *P. aeruginosa* [16,20,21,32,49,50,125] and *K. pneumoniae* [15,29]. Thus, the presence of these two VOCs alone cannot be used to confirm the presence of *E. coli*. A similar situation has been documented with acetic acid [21,49], the product of anaerobic respiration of carbohydrates, as this VOC has also been identified in the HS of *P. aeruginosa* cultures [20]. Heavier ketones, such as 2-tridecanone [21,35] and 2-pentadecanone [21,35], and alcohols, such as 1-octanol and 1-hexadecanol [16,21,35], are also interesting cases, as they have only been reported for *E. coli* thus far.

The profile of volatiles emitted by *S. aureus* includes 3-methylbutanoic acid (isovaleric acid) [16,22,44,48], ethyl 2-methylbutyrate [16,48], and 3-hydroxy-2-butanone (acetoin) [16,21,44,128]. All these compounds originate from one of two primary metabolic pathways: 3-methylbutyric acid is a product of amino acid (leucine) metabolism, while ethyl 2-methylbutyrate and acetoin are derived from different stages of the fermentation process in glucose metabolism [125,129]. Isovaleric acid is highly related to the generation of human body odor [21,130] and is also known as the characteristic VOC emitted by various *Staphylococcal* spp. [131]; as such, it has the potential to differentiate the etiology

of HAIs. Although acetoin has been identified in four independent studies focusing on *S. aureus* [16,21,44,128], it cannot be defined as species-specific, as it was also found in studies on *E. coli* [21], *K. pneumoniae* [46], and *P. aeruginosa* [72], while the production of ethyl 2-methylbutyrate was reported for *P. aeruginosa* by Karami et al. [41]. Thus, isovaleric acid is the only *S. aureus*-specific VOC to have been identified to date.

The volatile metabolites most often reported for *P. aeruginosa* have been mainly attributed to lipid oxidation pathways (i.e., acetone [12,24,27,28,33,34,39,55,72] and 2-butanone [12,24,26,27,39,49,50,55,72,125]) as the products of decarboxylation of β-keto acids [59]. Both acetone and 2-butanone have also been detected in other bacteria culture HSs. The production of acetone has been observed in studies focusing on *E. coli* [14,19,28,34,57] and *S. aureus* [12,28,49], while 2-butanone has been identified in analyses of *E. coli* [14,19,21,50,57], *S. aureus* [21], and *K. pneumoniae* [15,49]. In addition, other ketones observed for *P. aeruginosa*, including acetophenone [37,39,55] and 2-undecanone [16,20,21,31,37,72], have also been found in the headpace of *E. coli* cultures [14,16,21,44]. Nevertheless, a large group of oxygenated compounds has been found to be characteristic to *P. aeruginosa*, including 2-nonanol [16,21], 2-undecanol [16,21,37], acrolein [53,59], 3-methyl-2-pentanone [39,72], methyl 2-methylbutanoate [53,55,72], and ethyl *tert*-butyl ether [33,72]. Another compound of interests is 1-undecene [16,20,21,31,32,37,49,50,52,72,125], which is most likely the product of fatty acid degradation through the β-oxidation pathway [50]. Although 1-undecene has been reported for *P. aeruginosa* in as many as 11 independent experiments, its usefulness as a unique biomarker is uncertain, as Gao et al. [2] detected its presence in the HS of an *Acinetobacter baumannii* (*A. baumannii*) culture. Unlike other HAI-related pathogens, only *P. aeruginosa* is able to produce cyanide compounds, including hydrogen cyanide (HCN) [12,31,32,55] and methyl thiocyanate [12,23,55,72], which are synthesized from HCN via an enzymatic pathway. The production of these compounds depends on growth conditions, the most important of which being decreased oxygen content and increased cell density, which may be present in the case of lung infections [55]. Other metabolites characteristic to *P. aeruginosa* include volatile nitrogen-containing compounds (VNCs) such as pyrrole [16,21,39,53], ammonia [12,55], 2-methyl 1-*H*-pyrrole [16,21], 5-methyl-2-heptanamine [31,32], and 5-methyl-2-hexanamine [31,32]. The occurrence of pyrrole in the HS of *K. pneumoniae* was reported by Rees et al. [46]. Similarly, 2-aminoacetophenone (2-AAP) [12,16,55], which is responsible for the "grape-like" odor of *P. aeruginosa* cultures [55], has been observed in the HS of *E. coli* [35] and *S. aureus* [12] cultures. The species-specific markers of *P. aeruginosa* consist of 19 VOCs, including the aforementioned cyanide, nitrogen, and oxygenated compounds, as well as a large range of hydrocarbons such as decane [21,39], tridecane [21,27], 1-nonene [37,125], 1,10-undecadiene [37,125], and 1-dodecene [37,125].

The predominant VOCs produced by *K. pneumoniae* are ketones, especially odd-carbon methyl ketones such as 2-heptanone [15,29,41,46] and 2-nonanone [15,29,52], which likely originate from the β-oxidation of fatty acids [15,29]. However, as both of these compounds have also been detected in cultures of other bacteria, they should be considered as fundamental byproducts of bacterial metabolism [29,49], rather than specific biomarkers of *K. pneumoniae* infection. For instance, 2-heptanone has also been detected in the HS of *E. coli* [14,21,34,38,44], *P. aeruginosa* [37,39,41,49,55,72,125], *A. baumannii* [41], and *S. aureus* [21,38] cultures, while 2-nonanone production has been reported for *E. coli* [16,21,35,44,52] and *P. aeruginosa* [16,20,21,31,32,37,52,72,125]. Other findings have indicated the release of diketones, including 2,3-hexanedione [41,46] and 2,3-butanedione [46,49] in *K. pneumoniae* cultures [15,46]. 2,3-butanedione can be obtained enzymatically via the oxidation of 3-hydroxy-2-butanone or directly and spontaneously from acetolactate [46]. However, none of the monoketones can be considered unique to *K. pneumoniae*. Since *K. pneumoniae* quickly develops resistance to antibiotics, it poses a serious threat to critically ill patients in intensive care units (ICUs); thus, further research aimed at identifying *Klebsiella pneumoniae*-specific VOCs for non-invasive diagnosis is urgently needed.

From a diagnostic point of view, bacteria with long periods of latent growth, such as MTB complex (*M. tuberculosis* and *M. bovis*) and non-tuberculosis *Mycobacterium* (NTM) (e.g., *M. avium, M. abscessus,* and *M. intracellulare*), can be particularly challenging. Given that NTMs are also important etiological factors in TB-like diseases that require specific treatment (NTMs are often unresponsive to first-line anti-TB therapies [43]), it is important to differentiate them from the MTB complex as quickly as possible. In this regard, 2-butylfuran has been suggested as a unique marker for infections caused by *M. avium* [17,36]. 6-methyl-5-hepten-2-one has been detected in *M. avium* cultures in two independent studies [43,54], as well as in a single culture of *K. pneumoniae* [41]. To date, species-specific VOCs have yet to be identified and confirmed in at least two independent studies for other pathogens in the *Mycobacterium* family. Regardless, Beccaria et al.'s [17] findings suggest that the detection of 2-methyl-3-(methylthio)-furan may be linked to the presence of *Mycobacterium* spp., and the presence of phenylacetaldehyde may be indicative of NTM infection.

3.2. Resistance-related diversity in VOC profiles

The growth of antimicrobial resistance represents a serious health concern. Several studies have provided interesting findings revealing that differences in antibiotic susceptibility, which depend on the bacteria's genotype, may be reflected in the VOC patterns produced by different strains within the same species [11,14,15,19,27,30,49,55,62,65]. Such observations have triggered further research into using the bacterial volatilome to quickly differentiate between resistant and susceptible strains. In addition, researchers have also investigated the genotype-phenotype relationship in an attempt to link the bacterial genome with volatile metabolites, particularly in terms of antimicrobial resistance.

In 2012, Shestivska et al. [55] were the first to document the wide range of VOCs emitted by genetically distinct, antibiotic susceptible and resistant strains of *P. aeruginosa* in sputum and blood samples from CF patients. Their findings revealed that no single compound can adequately serve as a biomarker for the presence of *P. aeruginosa*; rather, the presence of this bacteria is best determined based on a group of several metabolites, including HCN, methyl thiocyanate, 2-AAP, acetophenone, 2-butanone, and methanethiol. In further studies by Boots et al. [49] and Bean et al. [62], VOC patterns were used to differentiate methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains. Seven of the eight discriminating VOCs showed increased levels in the MRSA samples (toluene, 2-pentanone, 2-heptanone, 2-dodecene, 1,4-dichlorobenzene, trichloro-acetic acid, and 2,2,3,3,-tetramethylhexane), while the only volatile indicating for the presence of MSSA strain was 1,1,2,2-tetrachloroethane [49]. Their findings were later confirmed by Li and Zhu [11], whose analysis of VOC profiles via SESI-MS enabled a clear distinction between MSSA and MRSA. Notably, Li and Zhu [11] also observed that the VOC profiles of these two species became less distinct from one another when treated with ampicillin, which is a finding that merits further investigation. Recent works by Drabińska et al. [14] and Hewett et al. [19] examining resistant strains of *E. coli* that produce extended-spectrum beta-lactamase (ESBL) have revealed statistically

significant differences between the VOC profiles of CTX-M beta-lactamase positive and negative *E. coli*. The abundance of bacterial fermentation product, butanoic acid, was higher in the non-CTM-X *E. coli* whereas the abundance of 2-dodecanone was higher in the resistant strains. Their results showed that these differences can be observed as quickly as 2 h after the addition of cefotaxime and cefepime, thus creating the potential for the rapid detection of antibiotic-resistant bacteria. Similar results were obtained by Smart et al. [27] in their study of three UTI-associated pathogens: *K. pneumoniae*, *P. aeruginosa,* and *E. coli*. Here, Smart et al. [27] observed significant differences in the VOC profiles of cephalexin and ciprofloxacin sensitive and resistant strains of *E. coli* and *K. pneumoniae* isolates less than 30 min after inoculation. Dodecanal and carbon dioxide have been shown to be more abundant in sensitive isolates without antibiotic addition, as have methyl ethyl sulfide, diethyl disulfide, and undecanal with antibiotic addition.

Due to its ability to mutate rapidly, many recent studies have investigated the antimicrobial resistance of *K. pneumoniae.* In 2018, Rees et al. [30] presented an innovative approach for the identification of carbapenemase-producing (CP) and non-CP strains of *K. pneumoniae* and *Enterobacter cloacae* based on the analysis of VOCs produced in *in vitro* cultures. The volatile metabolic patterns determined using SPME-GC × GC-TOF-MS enabled the authors to distinguish between CP and non-CP isolates with an area under the receiver operating characteristic curve (AUROCC) of 0.840 and 0.912 for the pan and core metabolomes, respectively (Table 1). These results were consistent with those obtained via matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), thus indicating the proposed protocol's usefulness for the detection of multidrug-resistant pathogens based on VOC analysis. Three years later, Wen et al. [65] analyzed carbapenem-resistant *K. pneumoniae* (CR-Kp) in infected plasma, with results showing significantly altered profiles of VOCs (i.a., *N*-acetyl glucosamine and butanoic acid) originating from the metabolism of carbohydrates, organic acids, fatty acids, and amino acids compared to samples infected with non-CR-Kp strains. The authors also observed significant changes in the emission of a newly discovered metabolite of *K. pneumoniae* (i.e., myoinositol) between CR-Kp and non-CR-Kp, which shall be the focal point of further research. In 2022, Filipiak et al. [15] performed a comprehensive analysis of VOCs produced by carbapenem-resistant and susceptible *K. pneumoniae* under normal and stressed conditions (i.e., addition of imipenem). One of the most striking observations in this study was that, compared to the sensitive strain, the carbapenem-resistant strain released significantly higher amounts of odd-carbon methyl ketones, including 2-pentanone, 2-heptanone, and 2-nonanone, most likely as a result of the β-oxidation of fatty acids.

3.3. Confounding factors for analyzing VOCs in vitro

Numerous studies have addressed the factors that influence bacterial growth and VOC production, including incubation temperature and time, and nutrient availability. If the emission of VOCs from human material (i.e., specimens isolated from patients, such as BAL fluid) is studied instead of cultures after a few passages, the most prominent influencing factor will be the storage conditions. Indeed, findings have shown that any changes during storage can impact on the number of VOCs detected [45]. As the storage time of the sample increases, the VOC profiles become more and more complex, which may result from sample contamination or degradation, residual bacterial activity, or a combination thereof [45,132]. Hence, the sample-storage conditions, especially the duration and temperature, should be defined when reporting the volatilome of thawed specimens.

The medium is another important component in bacteria cultivation, as it provides the nutrients for metabolic processes. To date, studies have shown significant differences in VOCs produced by a given species grown on different media [16,18,29,35,37,38,50,54,56]. The greatest discrepancies, especially in the production of alcohols, ketones, and acids, can be observed when comparing glucose-containing media (brain-heart infusion (BHI) and tryptic soy broth (TSB)) and glucose-free (Luria-Bertani broth (LB)) [16], as the latter produce smaller quantities of primary metabolites. There is also evidence that the emission of VOCs depends on the selected medium-sterilization method [50]. As shown by Rees et al. [29] for *K. pneumoniae,* a set of defined compounds is produced by the bacteria regardless of the type of growth media, constituting the "core" volatilome for certain species. They hypothesized that these compounds are likely the products of pivotal metabolic pathways, with the differences in their quantities being due to the varying availability of carbon in the environment [29]. A discrepancy in the emission of VOCs can also be observed between cultures in standard media and whole human blood [46,51]. For instance, Umber et al. [51] concluded that changing the LB medium to whole human blood resulted in significant changes in the volatile profile of *E. coli*.

It is also important to consider the growth phase in which bacteria sampling takes place when comparing the volatilomes of certain strains determined in unrelated studies. Typically, the bacteria growth curve consists of four distinct phases: the lag phase, the logarithmic phase (exponential), the stationary phase, and the death phase. Since the quantity and portfolio of VOCs both change depending on the growth phase, one should pay particular attention to the selection of "biomarkers" for pathogen identification, especially if applied to clinical settings, and ultimately, the choice of treatment. The VOC patterns observed in the stationary phase are typically more complex and stable compared to those in the exponential phase, which is partly due to the accumulation of volatiles produced in both phases [45,46]. In addition to the growth phase, the incubation temperature may induce quantitative and qualitative changes in the production of VOCs. For instance Timm et al. [37] observed that culturing *P. aeruginosa* at 37 °C rather than 30 °C yielded higher concentrations and longer-chain VOCs (especially 2-undecanone and 2-undecanol). Another factor that may influence the metabolism of VOCs is whether the bacteria are cultured under aerobic or anaerobic conditions. This was demonstrated by Koehler et al. [20], who found significant differences between *P. aeruginosa* biofilms cultured in the presence or absence of oxygen.

3.4. Co-cultures

It is well-documented that pathogenic microorganisms coexist with a range of other cells, such as fungi and human host cells (particularly immune cells), at the site of infection. Notably, these microorganisms can exhibit both competitive and, under appropriate circumstances, synergistic behavior with other cells and etiological factors. For example, while *S. aureus* is the dominant pathogen in the respiratory tracts of early childhood CF patients, it is gradually displaced by *P. aeruginosa* during the onset of adolescence [133]. To understand how the interactions between coexisting species affect the overall volatilome, several research groups performed *in vitro* experiments using mixed cultures (co-cultures). The results of these studies are discussed below.

Filkins et al. [133] observed that *S. aureus* and *P. aeruginosa* coexist during initial growth in co-culture, but that *P. aeruginosa* reduces the viability of *S. aureus* within 10–16 h of cultivation due to the production of siderophores (pyoverdine and pyochelin) and 2-heptyl-4-hydroxyquinoline-*N*-oxide. Interestingly, genetic mutant strains of *S. aureus*, so-called small-colony-variants (SCV) characterized by defects in their electron transport chain, were

killed to a lesser extent, suggesting that SCVs may comprise the more resilient strains of *S. aureus*. In findings reported by Chen et al. [44], the growth of *S. aureus* was significantly suppressed by *E. coli* during the lag and early logarithmic growth phases, indicating much earlier competition between these two species. This relationship was reflected in the significant reduction of the profiles of 3-methylbutanoic acid (reported often as isovaleric acid) and 3-methylbutanal, which are characteristic VOCs for *S. aureus*, and the near lack of change in indole, which is a characteristic marker of *E. coli*.

Lawal et al. [33] examined co-cultures of *P. aeruginosa* and *Enterobacter cloacae* in the presence of transformed lung epithelial cells (A549) to determine whether putative interactions between them affects the secretion of VOCs. Apart from the compounds characteristic to each species when tested alone, the co-cultures with *Enterobacter cloacae* also emitted 2-methylbutyl acetate, which may be useful in distinguishing between mono- and poly-microbial infections of *P. aeruginosa* [34]. Lawal et al. [33] observed that the levels of VOCs in the co-culture were significantly higher than the sum of both monocultures, leading them to conclude that the overproduction of these metabolites was intended to suppress the growth of competitive bacteria and establish the dominance of one of the co-cultured species. Although both *tert*-butyl ethyl ether and *tert*-butyl methyl ether were detected only in bacterial co-cultures with A549 cells [33], their classification as markers of *P. aeruginosa* interspecies interactions should be verified further, as there is evidence that both compounds are simply released by the A549 cells [134].

Multi-pathogen infections are particularly frequent in CF patients. Such cases often involve initial infection with a respiratory syncytial virus (RSV), which in turn triggers a host immune response that promotes the formation of *P. aeruginosa* biofilm, thus exacerbating the disease [135]. To better understand this process, Purcaro et al. [24] sought to analyze the volatile signatures of co-cultures consisting of cystic fibrosis bronchial epithelial (CFBE) cells, *P. aeruginosa*, and RSV. However, their results were unable to distinguish between *P. aeruginosa*+/RSV+ and *P. aeruginosa*+/RSV− infections, as the VOC patterns in the co-culture were dominated by volatiles originating from *P. aeruginosa*.

3.5. VOC release/uptake kinetics

It is assumed that the dynamic changes in the kinetic profiles of VOCs produced by bacteria in monocultures depend either on their temporary metabolic activity, or on their total load of bacterial cells. In the first case, the production rate of certain VOCs is highest when bacterial cell division is at its biological maximum (roughly in the middle of the logarithmic phase), followed by reduced production thereafter. In the second case, the VOC concentration profile is either directly proportional to the absolute content of bacteria cells in the culture (VOC secretion curve is similar to a growth curve) or inversely-proportional to the bacteria load if they are consumed as energy or substrate source (VOCs exhibiting continuously decreasing profile over the course of bacteria growth). Such knowledge relating to the dynamic changes in VOC profiles could be useful for clinical practice in two ways: firstly, metabolites released in the early phase of microbial growth, although only temporarily, may reflect an emerging infection, thus indicating the need for the rapid implementation of targeted therapy; and secondly, compounds whose secretion is directly proportional to the total burden of bacterial cells could be monitored over the course of infection, thus reflecting the effectiveness of the applied antibiotic treatment. Such dynamic changes in the emission of VOCs has been recently demonstrated for carbapenem resistant and susceptible strains of *K. pneumoniae* [15].

Similar to the qualitative discrepancies, quantitative VOC secretion can differ between bacteria species. This was shown by Filipiak et al. [125], who observed clear catabolism of aldehydes by *P. aeruginosa*, but strong production by *S. aureus* in the function of their growth time. Moreover, significant differences in kinetics (e.g., early release 1.5−3 h after inoculation) for ketones, hydrocarbons, alcohols, esters, volatile sulfuric compounds, and VNCs were also observed for these species. Elsewhere, Fitzgerald et al. [16,21] observed elevated secretion of alcohols between 8 and 24 h of *S. aureus* growth; however, after the 24 h mark, alcohol production ceased and the production of acids increased. The research by Chen et al. [44] indicates that the highest release of volatile metabolites from bacteria typically occurs 6−10 h after inoculation. These findings are further supported by Zhong et al. [25] and Karami et al. [38], who observed a remarkable increase in indole concentrations during the early growth phase of *E. coli*.

4. Animal models

The most recent animal studies of volatile metabolites related to bacterial infections (summarized in Table 2) have largely focused on lung diseases such as TB [61] or pneumonia, mainly caused by *S. aureus* [40,62,63] and *P. aeruginosa* [40,59,60,63]. The initial studies in 2013 and 2015 demonstrated the feasibility of using SESI-MS to analyze exhaled breath from mice infected with different bacteria. Both, Zhu et al. [63] and Bean et al. [62] have observed the same "features" for the mice infected with *S. aureus*, namely *m*/*z* 75, 88, 101, and 119. Although these authors were able to successfully differentiate subpopulations of animals based on "breathprints" characteristic to particular etiological factors, the volatile metabolites enabling this distinction remained unidentified [62,63]. Based on the obtained *m*/*z* values, it may be hypothesized that two of the features could indicate the presence of isovaleric acid (*m*/*z* 119) and acetoin (*m*/*z* 88), which were also noted in *in vitro* studies on *S. aureus* [16,44]. In 2018, Franchina et al. [61] applied TD-GC-MS to analyze breath samples collected from mice infected with MTB complex, with results revealing only one statistically different feature (i.e., decyl-cyclohexane) between the infected and control groups. In 2017, Zhou et al. [40] applied SPME-GC-MS for breath analysis to develop a new model for the fast and non-invasive diagnosis of infections caused by *E. coli*, *P. aeruginosa*, and *S. aureus* in rabbits. In 2019, van Oort et al. [60] compared the abilities of SIFT-MS and GC-MS to analyze the compositions of breath samples collected from rats with pneumonia, finding that only GC-MS was able to discriminate between infected and control animals [60]. Using this analytics platform, they identified over a dozen volatile organic compounds produced mainly by *Streptococcus pneumoniae*. Intriguingly, hexadecane was linked in above-mentioned works to lung infection, regardless of the underlying pathogen and animal (*E. coli*, *P. aeruginosa*, and *S. aureus* in rabbits [40], and *Streptococcus pneumoniae* in rats [60]), while 2,4-dimethylheptane was previously linked to *S. aureus* and *E. coli* infection in humans [79]. Finally, Purcaro et al. [59] employed sorption tubes followed by comprehensive GC × GC-TOF-MS analysis to evaluate the core breath metabolome characteristics for murine *P. aeruginosa* infections. Two out of the identified VOCs were previously reported as related to the presence of *P. aeruginosa* genus, namely 2-hexanone [72] and cyclohexanol [136]. According to Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the latter may be derived from aromatic compounds degradation or microbial metabolism in diverse environments pathways and has been reported as bioactive agent on fungi [136]. 2-hexanone was significantly more abundant in the breath of mice infected with ultraviolet-killed *P. aeruginosa* and control group compared to the live-*P. aeruginosa* infected group, which shows the probability of

Table 2
Breath analysis in the animal model.

Group/sample matrix	Pathogen	Analytical platform	Aims	Observations	Refs.
48 mice/breath	*P. aeruginosa* (PAO1, PA14, PAK, and PA7)	TD-GC × GC-TOF-MS (Tedlar bags and sorbent tubes)	• Identify core VOCs from different strains of *P. aeruginosa.*	• 9 discriminative VOCs between mice inoculated with live *P. aeruginosa*, ultraviolet-killed *P. aeruginosa*, and PBS. • 5 VOCs confirmed with analytical standards: isoborneol, *p*-cymene, 2-hexanone, 4-cyclopentene-1,3-dione, and cyclohexanol.	[59]
50 rats/breath	*P. aeruginosa and Streptococcus pneumoniae*	TD-GC-MS (sorbent tubes) and SIFT-MS	• Discriminate between infected and non-infected animals. • Discriminate between *S. aureus* and *P. aeruginosa* infection based on exhaled breath analysis. • Compare both analytical platforms.	• 8 VOCs discriminating infected animals and non-infected animals. • 14 VOCs discriminating the *S. aureus*-infected animals and the controls. • 3 VOCs discriminating the *P. aeruginosa*-infected animals and the controls. • Both GC-MS and SIFT-MS provided adequate accuracy (AUROCC > 0.7) to differentiate between causative pathogens. • Only GC-MS could discriminate between infected and non-infected animals (AUROCC of 0.85 for GC-MS and 0.54 for SIFT-MS).	[60]
16 mice/breath	*Mycobacterium bovis* Bacillus Calmette-Guerin	TD-GC × GC-MS (Tedlar bags and sorbent tubes)	• Develop a breath analysis method for differentiating between healthy and *Mycobacterium bovis*-infected animals.	• A panel of 23 VOCs differentiating infected and non-infected mice (11 more abundant in *Mycobacterium bovis*-infected, 8 more abundant in control, and 4 in room air samples).	[61]
Unknown number of rabbits/breath	*E. coli* (ATCC 25922) *S. aureus* (ATCC 25923) *P. aeruginosa* (ATCC 27853)	SPME-GC-MS	• Determine exhaled characteristic VOCs of most prevalent pathogens of nosocomial pneumonia to enable rapid identification.	• Bacterial pneumonia VOCs: 1H-pyrrole-3-carbonitrile, diethyl phthalate, cedrol, decanoic acid, cyclohexane, *trans*-squalene, diisooctyl phthalate, and heptasiloxane. • 2 VOCs specific to *S. aureus*: decanoid acid and 1*H*-pyrrole-3-carbonitrile.	[40]
12 mice (6 for MSSA and 6 for MRSA)/ breath	MRSA (450 M) MSSA (RN450)	SESI-MS (Tedlar bags)	• Develop a rapid and non-invasive method for discriminating between MSSA and MRSA lung infections *in situ*.	• 25 infection-related significant features (14 for MSSA and 11 for MRSA).	[62]
6 mice per group/ breath and BAL	*K. pneumoniae* (ATCC 13883)*P. aeruginosa* (PAO1-UW)*S. aureus* (RN450)[a]	SESI-MS (Tedlar bags)	• Assess the utility of SESI-MS breathprints for diagnosis by applying it for the analysis of breath samples from mice with lung infections.	• 90 discriminative features between all bacteria. • Highly unique and reproducible patterns for each bacterium. • 2 features specific to *K. pneumoniae.* • 1 feature specific to *S. aureus.*	[63]

[a] Authors also investigated other bacteria that were beyond the scope of this review. *P. aeruginosa*: *Pseudomonas aeruginosa*; TD: thermal desorption; GC × GC-TOF-MS: two-dimensional gas chromatography time-of-flight mass spectrometry; VOCs: volatile organic compounds; PBS: phosphate-buffered saline; SIFT: selected ion flow tube; *S. aureus*: *Staphylococcus aureus*; AUROCC: area under the receiver operating characteristic curve; *E. coli*: *Escherichia coli*; SPME: solid-phase microextraction; MSSA: methicillin-susceptible *S. aureus*; MRSA: methicillin-resistant *S. aureus*; SESI: secondary electrospray ionization; BAL: bronchoalveolar lavage; *K. pneumoniae*: *Klebsiella pneumoniae*.

uptake of this compound by *P. aeruginosa*. Their findings indicated that the proposed method was able to distinguish between healthy and infected mice with an accuracy of 81% [59].

5. Bacterial VOCs for disease diagnosis

Over the past 10 years, the possibility of using bacterial VOC detection to enable the rapid, non-invasive diagnosis of diseases has been extensively investigated. While analyses of various human materials (e.g., urine, blood, breath, tissues, pus, and swabs) with adequately modified analytical platforms have shown the great potential of this approach, clinical studies have largely focused on diverse types of cancer. In this section, we provide an overview of recent applications of volatilome analysis in the clinical diagnosis of bacterial infections (summarized in Table 3).

5.1. Respiratory infections

Lower respiratory tract infections (LRTI) are one of the most common causes of morbidity and mortality in humans, particularly in mechanically-ventilated patients and those suffering from chronic respiratory diseases [2,13,15,70,71]. For both acute (VAP) and recurrent bacterial infections in patients with chronic diseases (CF, TB, and chronic obstructive pulmonary disease (COPD)), it is critical to detect the causative pathogen as early as possible so as to enable the most timely implementation of adapted therapy. The majority of studies on VOCs in the field of respiratory diseases have been performed using breath samples, as they are readily available and can be collected in a simple and non-invasive manner.

5.1.1. VAP

VAP is a common hospital-acquired infection that develops in mechanically ventilated patients and is characterized by a very high mortality coefficient (30%–70%) [137]. Recent volatilomic studies have demonstrated that breath analysis can be a feasible complement to current methods of VAP diagnosis and pathogen identification. In 2015, Schnabel et al. [80] used a set of 12 VOCs to correctly discriminate between VAP and non-VAP patients with accuracy, sensitivity, and specificity values of 74.2%, 75.8%, and 73%, respectively. Their study included 100 ventilated patients, which remains

Table 3
Applications of volatile organic compounds profiling in various matrices for diagnosing in clinical conditions.

Type of infection	*In vivo*/*ex vivo* sample matrix	Analytical platform	Group	Pathogens	Aims	Observations	Refs.
Central nervous system infections	*Ex vivo*, cerebral-spinal fluid	HS-SPME-GC-MS	58 samples from patients with ME (16 with bacterial ME and 42 with viral ME)	No data (differentiating bacteria vs viruses)	• Search for potential biomarkers enabling differentiation between bacterial and viral ME in CSF.	• Ethylene oxide levels were significantly higher in the bacterial ME group vs. the viral ME group. • Phenol levels were significantly higher in the viral ME group vs. the bacterial ME group.	[64]
Vascular graft infections, endovascular infections, infective endocarditis, bone and prosthetic joint infections	*Ex vivo*, lung tissue, blood, nasal aspirate, cardial device or heart valve	SESI-HRMS	17 samples	MSSA and MRSA[a]	• Evaluate the technical feasibility of SESI-HRMS as a diagnostic tool for the rapid detection of bacterial infections.	• High-level sensitivity (1000 CFUs ↓) and great selectivity driven by the high resolution enabled by the mass analyzer. • Total analysis times of five-ten minutes to fingerprint samples. • Hundreds of *S. aureus*-specific features.	[10]
Wound infections	*Ex vivo*, wound swabs	GC-IMS	19 samples	*S. aureus P. aeruginosa*[a]	• Evaluate the ability of GC-IMS to differentiate between infected wounds and colonised wounds.	• Six discriminative features distinguishing infected wounds from colonised wound. • Sensitivity reaching 100% and specificity of 88% (Random Forest).	[8]
External otitis	*Ex vivo*, pus	GC-MS LuxR receptor (biosensor)	26 individuals	*P. aeruginosa*	• Evaluate a LuxR-harboring whole-cell biosensor strain as a new tool for the specific detection of *P. aeruginosa* otitis, via the binding of 2-aminoacetophenone.	• Results obtained by the biosensor matched the results from culture assays (92%) and GC-MS analysis (96%).	[4]
Sepsis	*Ex vivo*, blood	Extraction, derivatization GC × GC-TOF-MS	38 samples (20 infected by carbapenem-resistant *K. pneumoniae*)	*K. pneumoniae*	• Analyze the metabolomes of CR-Kp-infected patients.	• 58 VOCs associated with CR-Kp infection. • Significant VOCs in CR-Kp infection: *N*-acetyl glucosamine, butanedioic acid, and myoinositol.	[65]
Sepsis	*Ex vivo*, blood	EI-MS and IMR-MS	152 aerobic (including 27 Gram-negative, 106 Gram-positive and 19 fungi) and 130 anaerobic (including 37 Gram-negative, 91 Gram-positive and two fungi) blood cultures from patients	*S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*[a]	• Analyze VOCs to differentiate microorganisms into Gram-positive and Gram-negative variants.	• For the training data set ($n = 86$): sensitivity (proportion of Gram-positives truly classified as Gram-positive) of 97.5% and specificity (proportion of Gram-negatives truly classified as Gram-negative) of 74.8%. • For the validation data set ($n = 42$): sensitivity of 93.3% and specificity of 58.3%. • Lack of VOCs identification	[66]
Sepsis	*Ex vivo*, blood	Extraction in bead mill, derivatization GC-TOF-MS	122 samples (73 with sepsis and 49 controls)	Not specified	• Analyze VOCs for the prediction of bacteremic sepsis.	• Predictive model for bacteremia based on six metabolites: myristic acid, citric acid, isoleucine, norleucine, pyruvic acid, and phosphocholine. • Sensitivity of 91% and specificity of 84%. • Myristic acid identified bacteremia with better accuracy than CRP (81.8% vs. 76.4%).	[67]

Sepsis	*Ex vivo*, blood	APCI-MS	130 samples (61 non-diagnosed, 39 diagnosed, and 30 healthy controls)	*S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *A. baumannii*	• Evaluate the effectiveness of APCI-MS for the analysis of VOCs in bacteremia patients to differentiate between causative pathogens.	• VOCs characteristic to *S. aureus*: butyric acid, isovaleric acid. • VOCs characteristic to *E. coli:* indole. • VOCs characteristic to *P. aeruginosa*: 1-vinyl aziridine • 33 successfully identified cases of infection (14 of *S. aureus*, 5 of *E. coli*, 7 of *K. pneumoniae*, 4 of *A. baumannii*, and 3 of *P. aeruginosa*).	[68]
Urinary tract infections	*Ex vivo*, urine	eNose sensor (based on IMS principle)	101 urine samples (21 of *E. coli*, 19 of *Staphylococcus saprophyticus*, 20 of *Klebsiella* species, and 21 of *Enterococcus faecalis)*	*E. coli Klebsiella species*[a]	• Evaluate the effectiveness of the IMS-based eNose sensor for the analysis of VOCs to discriminate the causative pathogens of UTIs.	• Sensitivity of 95% and specificity of 97% in the discrimination of sterile from bacterial samples. • Sensitivity of 95% and specificity of 96% in the identification of bacterial species using eNose vs. urine bacterial cultures.	[7]
Infections in CF	*Ex vivo*, BAL	HS-SPME-GC × GC-TOF-MS	154 samples	*P. aeruginosa* and *S. aureus*	• Analyze VOCs in BAL samples.	• 9 VOCs discriminating between *P. aeruginosa*+ and *P. aeruginosa*- cases (AUROCC of 0.86). • 8 VOCs discriminating between *S. aureus*+ and *S. aureus*- cases (AUROCC of 0.88). • 5 VOCs to *P. aeruginosa*: 2-methyl-2-butanol ↑, 3-methyl-2-butanone/1-butanol ↓, 2-butanol, ethyl acetate. • For *PA*, *S. aureus*, and *S. aureus* + *P. aeruginosa*: 2-butanone ↑.	[69]
Infections in CF	*In vivo*, breath	TD-GC-MS (Tedlar bags and sorbent tubes)	18 individuals (13 with *S. aureus* infection and 5 without)	*S. aureus*	• Discriminate between *S. aureus*-infected and non-infected CF patients based on the analysis of VOCs in exhaled breath samples.	• Nine VOCs significant for discriminating *S. aureus*-infected from non-infected CF patients: 1,4-pentadiene ↑, acetone, undecane, ethanol, 2-butanone, 2-methyl-naphtalene/3-hydroxy-2-butanone ↓, hexanal, isopropyl myristate. • Sensitivity of 100%, specificity of 80%.	[70]
Infections in CF	*In vivo*, breath	Bio-VOC® sampler, followed by SPME-GC-MS	Nine CF patients	*S. aureus* and *P. aeruginosa*	• Analyze the composition of exhaled VOCs for the rapid identification of causative pathogens in patients with LRTI.	• 33 signals specific for breath samples (2 detected in all samples) • 100% accuracy in the identification of CF patients and healthy individuals.	[47]
Infections in CF	*In vivo*, breath	SIFT-MS	233 pediatric CF patients (156 with no *P. aeruginosa* and 77 with *P. aeruginosa)*	*P. aeruginosa*	• Investigate whether HCN in exhaled breath is a biomarker of early *P. aeruginosa* infection in children with CF.	• Sensitivity of 33% and specificity of 99% for the identification of HCN in breath.	[13]
Infections in CF	*In vivo*, breath	SIFT-MS	20 individuals (10 with chronic *P. aeruginosa* infection and 10 with no *P. aeruginosa*)	*P. aeruginosa*	• Investigate whether HCN in exhaled breath (oral and nasal) is a biomarker for *P. aeruginosa* infection in adults.	• HCN concentrations in nasal exhaled breath were significantly higher in patients with *P. aeruginosa* (11 ppbv vs. 0 ppbv).	[71]

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Table 3 (*continued*)

Type of infection	*In vivo*/*ex vivo* sample matrix	Analytical platform	Group	Pathogens	Aims	Observations	Refs.
						• Sensitivity of 60% and specificity of 80%.	
Infections in CF	*Ex vivo*, sputum	HS-SPME-GC-MS	28 samples	*P. aeruginosa*	• Analyze VOCs in the HS of sputum samples to predict *P. aeruginosa* infection (*P. aeruginosa* model) or *P. aeruginosa* chronic colonization (PACC model) in CF patients.	• For *P. aeruginosa* model: sensitivity of 72% and specificity of 40%. • For PACC model: sensitivity of 100% and specificity of 67%. • Positive correlation with *P. aeruginosa* presence: 1-undecene, 1-α-pinene, dodecane, terpinen-4-ol, and 2,2,6-trimethyl-octane. • Negative correlation with *P. aeruginosa* presence: dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, hexane, and 2-methyl-pentane.	[72]
Infections in CF	*Ex vivo*, sputum	HS-SPME-GC-MS	72 samples (13 CF patients, 59 non-CF): 32 *P. aeruginosa* positive, 12 no pathogens, and 28 other pathogens	*P. aeruginosa*	• Determine VOCs specific to *P. aeruginosa* in sputum samples.	• Sputum library comprising 17 VOCs (sensitivity 62% and specificity 100%). • 2-nonanone as a single *P. aeruginosa* marker (sensitivity 72% and specificity 88%). • Combination of sputum library and *P. aeruginosa* increased the sensitivity to 91% with specificity of 88%.	[73]
Pulmonary tuberculosis	*In vivo*, sputum/breath	TD-GC × GC-TOF-MS (sorbent tubes)	50 individuals (32 with active pulmonary TB and 18 control group with TB symptoms)	MTB	• Analyze human breath to identify active TB cases among suspected TB patients, considering several risk factors for TB for smokers and those with HIV.	• 23 discriminatory features for TB. • Accuracy of about 80%–90% for three models (RF, PLS-DA, and SVM), sensitivity of 85%–100%, and specificity of 60%–80%.	[74]
Pulmonary tuberculosis	*In vivo*, breath	eNose sensor	51 TB individuals and 20 controls	MTB	• Evaluate the suitability of eNose sensors for the diagnosis, prognosis, and monitoring of treatments for TB via breath analysis.	• For the discrimination of TB cases from controls: sensitivity of 94.1% and specificity of 90%. • Signals obtained on day 30 of TB therapy significantly overlapped with those for the controls.	[5]
Pulmonary tuberculosis	*In vivo*, breath	eNnose and Aenose® sensors	110 individuals (47 with TB, 14 with asthma/COPD, and 49 healthy controls)	MTB	• Evaluate the performance of Aenose® sensors for the diagnosis of TB via breath analysis.	• For the discrimination of TB cases from controls only: sensitivity of 91% and specificity of 93%. • For the discrimination of TB cases from asthma/COPD cases and controls: sensitivity of 88% and specificity of 92%.	[6]
Pulmonary tuberculosis	*Ex vivo*, urine	CSA	63 samples (22 TB samples and 41 TB suspect controls)	MTB	• Diagnose TB via urine HS analysis using a CSA.	• 14 important indicators for detecting TB's signature. • After 60 min: sensitivity 83.7%, accuracy 78.6%, and specificity 76.2%.	[75]
Pulmonary tuberculosis	*In vivo*, breath	FAIMS	40 individuals (21 with suspected TB and 19 healthy controls)	MTB	• Evaluate the effectiveness of FAIMS for analyzing breath	• For the discrimination of all types of TB from the healthy controls:	[76]

					samples from patients with pulmonary TB and extra-pulmonary TB.	sensitivity of 81% and specificity of 79%.	
Pulmonary tuberculosis	*In vivo*, breath	Thermal desorption and nanomaterial-based sensors	198 individuals (44 TB positive, 47 TB negative, 47 healthy, and 60 for blind validation; $n = 20$ for each group)	MTB	• Evaluate the performance of organically modified nanomaterial-based sensors for the active detection of TB via the analysis of exhaled breath.	• For the validation of known identity samples: accuracy of 92%, sensitivity of 90%, and specificity of 93%. • For blind validation: accuracy of 88%, sensitivity of 76%, and specificity of 94%.	[77]
VAP	*In vivo*, breath	TD-GC-MS (sorbent tubes)	93 individuals (47 control, 13 colonized, 21 possible VAP, and 12 probable VAP): 25 cultures positive, and 68 cultures negative	No data (differentiating VAP vs. non-VAP)	• Differentiate between VAP, colonized, and control samples via the analysis of VOCs in exhaled breath samples from intubated MV patients.	• 11 VOCs significantly altered for VAP samples (sevoflurane, hexafluoroisopropanol, fluorocarbon derivatives were exogenous). • 52 VOCs significantly different in colonized samples.	[78]
VAP	*In vivo*, breath	TD-GC-MS (sorbent tubes)	52 individuals	*A. baumannii*	• Analyze exhaled *A. baumannii*-derived VOCs to enable the early diagnosis of VAP and to discriminate between colonized and infected samples.	• Breathprint of eight VOCs *in vivo*: 1-undecene, nonanal, decanal, 2,6,10-trimethyl-dodecane, 5-methyl-5-propyl-nonane, longifolene, tetradecane, and 2-butyl-1-octanol. • Only 4 VOCs the same *in vitro* and *in vivo*: 1-undecene, decanal, longifolene, and tetradecane. • For the discrimination of *A. baumannii* samples (infection and colonization) from the controls: sensitivity and specificity of 83.3%. • For discriminating between infected and control samples: sensitivity and specificity of 85%.	[2]
VAP	*In vivo*, breath	TD-GC-MS (sorbent tubes)	28 individuals	*S. aureus*, *E. coli*, and *P. aeruginosa*[a]	• Non-invasive detection of VAP-causative pathogens via the analysis of VOCs in exhaled breath samples.	• Correlation between VOC profiles and VAP severity over the course of the disease. • For *S. aureus*: 12 of 32 VOCs previously reported to be released *in vitro* were also present in breath samples. • For *E. coli*: dimethylsulfide, pyrrole, acetonitrile, methanol, ethanol, acetaldehyde, 2-pentanone, and 2-methylfuran. • For *P. aeruginosa*: 1-nonene, 1-undecene, and dimethylsulfide.	[79]
VAP	*In vivo*, breath	TD-GC-TOF-MS (Tedlar bags and sorbent tubes)	100 individuals (32 VAP+ and 68 VAP-)	*S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *A. baumannii*	• Identify VAP-specific VOCs *in vivo* via the analysis of exhaled breath samples from critically ill MV patients.	• 12 VOCs discriminating VAP+ from VAP- samples. • VOCs present at elevated levels: 2-methylbutane ethanol, heptane, ethylbenzene, carane, tetradecane, and tetradecanal.	[80]

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Table 3 (*continued*)

Type of infection	*In vivo*/*ex vivo* sample matrix	Analytical platform	Group	Pathogens	Aims	Observations	Refs.
						• VOCs present at reduced levels: acetone isopropanol, acrolein, tetrahydrofuran, and dodecane. • Accuracy of 74.2%, sensitivity of 75.8%, and specificity of 73%.	
VAP	*In vivo*, breath	TD-GC-MS (sorbent tubes)	46 individuals	*S. aureus*[a]	• Metabolic profiling of exhaled breath samples to identify specific VOCs of pathogens associated with VAP in MV patients.	• For infected vs. non-infected patients: 3-carene ↑, *n*-butyric acid, 2-ethylhexyl ester, nonanal, and 2,6,11,15-tetramethyl-hexadecane; ethanol ↓, 2-methyl cyclopentanone, heptane, and *N*-cyclohexyl-*N*′(2-hydroxyethyl) thiourea.	[81]

[a] Authors also investigated other bacteria not included in this review (i.e. beyond of the ESKAPE group); ↑: elevated; ↓: reduced. MTB: *Mycobacterium tuberculosis*; HS: headspace; SPME: solid-phase microextraction; GC-MS: gas chromatography-mass spectrometry; ME: meningitis/encephalitis; CSF: cerebrospinal fluid; SESI-HRMS: secondary electrospray ionization-high resolution mass spectrometry; MSSA: methicillin-susceptible *Staphylococcus aureus* (*S. aureus*); MRSA: methicillin-resistant *S. aureus*; CFU: colony forming unit; GC-IMS: gas chromatography-ion-mobility spectrometry; *P. aeruginosa*: *Pseudomonas aeruginosa*; GC × GC-TOF-MS: two-dimensional gas chromatography time-of-flight mass spectrometry; CR-Kp: carbapenem-resistant *Klebsiella pneumoniae* (*K. pneumoniae*); VOCs: volatile organic compounds; EI: electron impact; IMR: ion−molecule reaction; *E. coli*: *Escherichia coli*; CRP: C-reactive protein; APCI: atmospheric-pressure chemical ionization; *A. baumannii*: *Acinetobacter baumannii*; IMS: ion mobility spectrometry; UTIs: urinary tract infections; CF: cystic fibrosis; BAL: bronchoalveolar lavage; AUROCC: area under the receiver operating characteristic curve;; TD: thermal desorption; LRTI: lower respiratory tract infections; SIFT: selected ion flow tube; HCN: hydrogen cyanide; TB: tuberculosis; HIV: human immunodeficiency virus; RF: Random Forest; PLS-DA: partial least squares-discriminant analysis; SVM: support vector machine; COPD: chronic obstructive pulmonary disease; CSA: colorimetric sensor array; FAIMS: field asymmetric ion mobility spectrometry; VAP: ventilator-associated pneumonia; MV: mechanically ventilated.

the largest cohort examined for breath-based VAP diagnosis. In the same year, Fowler et al. [81] showed that VOC patterns in exhaled breath from ventilated patients could be used to differentiate groups with respiratory pathogens from colonized and healthy controls [81]. Similarly, van Oort et al. [78] also used VOCs to discriminate VAP patients from non-VAP controls; however, some of the reported 11 VOCs were exogenous (sevoflurane, hexafluoroisopropanol, and fluorocarbon derivatives) and therefore should not be used for this purpose. Filipiak et al. [79] examined endogenous volatiles by collecting end-tidal breath samples from mechanically ventilated (MV) patients under capnography monitoring and prevented the loss of sample components (condensation of humid breath, VOCs displacement with water, etc.) by elevating the adsorption temperature on multibed tubes. They observed that the exhaled concentration profiles of specific volatile metabolites changed dynamically over the course of the bacterial infection, specifically in relation to the severity of the infection, as reflected by the concentration of C-reactive proteins and other patients' clinical conditions. A year later, Gao et al. [2] successfully used microbial VOCs determined in exhaled breath samples via TD-GC-MS to confirm the presence (or absence) of *A. baumannii* in the lower airways of ventilated patients, and to distinguish between the colonization of airways from acute VAP infection. It is worth noting that as many as four of the compounds identified *in vivo* (1-undecene, decanal, longifolene, and tetradecane) were also confirmed in *in vitro* analyses, which proves their great potential as markers of *A. baumannii* infection. Likewise, Filipiak et al. [79] found in end-tidal-air of patients with confirmed *E. coli* infection VOCs that have been reported by other research groups as volatile metabolites released by *E. coli* under *in vitro* conditions, i.e., dimethylsulfide (DMS) [14,19,28,34,51], ethanol [14,19,52,57], acetaldehyde [51,57] and 2-pentanone [28]. Taking into account the here discussed studies, the exhaled breath analysis is very likely to be suitable both for differentiating patients with VAP from healthy controls (AUROCC of 0.87 [80], 0.73 [78] and 0.88 [2]) as well as for predicting the presence of or absence of pathogens in the lower respiratory tract (AUROCC of 0.69 [78] and 0.89 [2]) with simultaneous identification.

5.1.2. TB

MTB is the main cause of TB, a life-threatening disease that can develop latently for a long time. Patients with latent TB have no typical symptoms and cannot transmit the infection to others, but 5%−10% of them, especially the immunocompromised, will develop active TB [138]. Since bacteria are alive but inactive in the latent phase of TB, they do not emit sufficient amounts of characteristic VOCs that could be transferred to extracellular space and, ultimately, exhaled breath. Therefore, the use of breath analysis to diagnose TB will be based on the active (infectious), rather than latent, form of the disease. Promising results have been reported by groups using eNose sensor arrays, which have enabled TB diagnosis with sensitivities and selectivities of 88%−94% and 90%−93%, respectively [5,6,77]. In addition to enabling the discrimination of TB patients from healthy individuals [5,6], breath analysis using eNose sensors has also proven to be useful in the monitoring of TB therapy [5]. In their work, Zetola et al. [5] demonstrated that eNose sensors can also be used to distinguish between patients with single pulmonary TB or co-infection of MTB and human immunodeficiency virus (HIV). In 2016, Lim et al. [75] successfully used a colorimetric sensor array (CSA) to differentiate patients with extrapulmonary TB from healthy ones based on urine sample analysis. In 2018, Beccaria et al. [74] applied GC × GC-TOF-MS to analyze breath samples from 50 donors in order to differentiate patients with confirmed MTB infection from those with one or more characteristic TB symptoms but a negative MTB diagnosis

[74]. Unlike Zetola et al. [5], they did not observe the effect of HIV co-infection on the protocol they developed to differentiate between MTB+ and MTB- patients, therefore this issue requires further research involving a larger study cohort. Analyzes carried out using GC × GC-TOF-MS (area under the curve (AUC) of 0.96) [74], field asymmetric ion mobility spectrometry (FAIMS) (AUC of 0.92) [76], and eNose sensors give hope that the analysis of VOCs of bacterial origin may become a rule-in or rule-out tool for TB diagnosis.

5.1.3. CF

CF is an inherited disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. CF patients frequently suffer from recurrent fungal and bacterial respiratory infections, mostly caused by *S. aureus* or *P. aeruginosa*. Gilchrist et al. [13,71] employed SIFT-MS to measure nose-exhaled breath samples from CF patients, with results confirming that chronically *P. aeruginosa*-infected and non-infected patients can be differentiated based on the analysis of hydrogen cyanide (previously reported *in vitro* for *P. aeruginosa* [12,31,32,55]) concentrations in their exhaled breath. Indeed, as discussed in the review by Smith et al. [139], hydrogen cyanide has been detected in both nasal and oral exhaled breath samples from CF patients and has been successfully used to diagnose early-stage *P. aeruginosa* infections, as well as to monitor the effectiveness of therapy. Similarly, Neerincx et al. [70] and Kramer et al. [47] analyzed breath samples via GC-MS to identify nine VOCs differentiating *S. aureus*-infected from non-infected CF patients. While some of the identified VOCs can be found in the exhaled breath of all individuals (e.g., ethanol, acetone, and 2-butanone), others have been proven to originate from *S. aureus* in previous *in vitro* experiments [125]. Although the applied analytical protocol enabled 100% sensitivity and 80% specificity in patient classification, the sizes of the cohorts in their study were very limited (10 persons with *S. aureus* on the day of breath sampling vs. 5 CF patients free from *S. aureus* infection) [47,70]. Hence, further studies are needed to confirm these findings.

In line with the increasing attempts to use breath samples to detect respiratory tract infections, *ex vivo* analyses have also been applied to determine bacterial metabolites. In recent years, several analytical approaches combining HS-SPME (the most popular being DVB/CAR/PDMS) with GC-MS have enabled the identification of etiological factors of infection [69,72,73]. In 2011, Savelev et al. [73] analyzed sputum samples from 72 patients (CF and controls), including 32 patients with confirmed *P. aeruginosa* infection. The results of this work would provide the basis for their "*Pseudomonas* sputum library," which consisted of 17 VOCs present in all *P. aeruginosa*-positive sputum samples (i.a., limonene, 2,4-dimethylheptene, and 3-methyl-1-butanol). In addition, they confirmed that the combination of this library and 2-nonanone in sputum allows for a more accurate distinction between infected and non-infected patients compared to the use of 2-nonanone on its own (sensitivity of 91% vs. 72%, respectively). Likewise, 4 VOCs mentioned above was also observed in sputum by Goeminne et al. [72] who analyzed samples from 28 individuals, with findings indicating that the presence of bacteria-specific VOCs allows not only the detection of acute *P. aeruginosa* infection, but also chronic colonization in CF patients (sensitivity of 100%). The authors also presented a set of 10 VOCs associated with the presence of *P. aeruginosa* in lungs, of which 1-undecene and dodecane [72] were the most significant. This result supports those reported in prior *in vitro* studies (Section 3.1). In 2018, Nasir et al. [69] coupled HS-SPME with GC × GC-TOF-MS to analyze 154 BAL samples from CF patients to identify the presence of *P. aeruginosa* and *S. aureus*. The proposed approach allowed them to successfully discriminate *P. aeruginosa*-positive from *P. aeruginosa*-negative samples using 9 VOCs (AUROCC of 0.86) and *S. aureus*-positive from *S. aureus*-negative samples using a combination of 8 VOCs (AUROCC of 0.88). The studies presented in this section indicate that the analysis of VOCs in relation to CF is certainly useful for revealing the presence of the pathogen and its recognition. However, further research is needed to confirm whether it is possible to differentiate between infection and chronic colonization in patients with CF.

5.2. Bloodstream infections

Bloodstream infection, also known as bacteremia, is a dangerous type of HAI and is the leading cause of septic shock. Sepsis can have mortality rates of up to 50%, even in patients being treated in modern, specialized centers, and these rates can worsen if targeted therapy is not implemented in a timely manner [140]. Therefore, researchers are intensively searching for bedside-compatible methods that can enable the prompt detection of the causative pathogen. To this end, the analysis of species-specific bacterial VOCs appears to be a particularly promising approach (see Table 1).

Regarding the ESKAPE pathogens, only 4 clinical trials involving patients with bloodstream infections have been performed during the last 10 years. In 2015, Chingin et al. [68] applied atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) for the analysis of VOCs in the HS of blood samples obtained from 61 bacteremia patients. Their findings indicated that infection with *S. aureus* and *P. aeruginosa* could be confirmed after as few as 10 h, with other bacteria from this group (*A. baumannii*, *K. pneumoniae*, and *E. coli*) being detectable by the 16 h mark [68]. Using the proposed approach, Chingin et al. [68] were able to identify 14 cases of *S. aureus*, 5 cases of *E. coli*, 7 cases of *K. pneumoniae*, 4 cases of *A. baumannii*, and 3 cases of *P. aeruginosa* infection. They confirmed the presence in biological samples of VOCs characteristic for pathogens *in vitro*: indole for *E. coli* as well as isovaleric acid and butyric acid for *S. aureus* (see Section 3.1). However, it remains unknown whether the obtained results indicated single- or multi-pathogen infections, as neither the selectivity nor sensitivity was given. In 2016, Kauppi et al. [67] analyzed blood sample extracts using a GC-TOF-MS-based method to differentiate between patients with and without bacteremia. They have identified the pattern of 6 metabolites which was more predictive for bacteremic sepsis (sensitivity, specificity, and AUROCC of 0.91, 0.84, and 0.93, respectively) than the best possible combination of four clinical variables, i.e., C-reactive protein, leukocyte count, thrombocyte count, and body temperature (sensitivity, specificity, and AUROCC of 0.82, 0.56, and 0.82). Out of the 6 detected sepsis-related metabolites, myristic acid emerged as being of particular interest. This compound indicated the presence of sepsis with an accuracy of 81.8%, which surpasses the accuracy of C-reactive protein (76.4%), one of the better current markers of an inflammatory state. In the same year, Dolch et al. [66] applied electron impact mass spectrometry (EI-MS) and ion-molecule reaction mass spectrometry (IMR-MS) to analyze blood cultures from 152 patients to differentiate between Gram-positive and Gram-negative bacteria. Despite being unable to identify characteristic VOCs, the authors developed a set of 10 discriminators that allowed Gram-positive and Gram-negative bacteria cultures to be distinguished from one another with sensitivity, specificity, and error rate values of 93.3%, 58.3%, and 16.7%, respectively. More recently, Wen et al. [65] successfully distinguished between CR-Kp and carbapenem-susceptible *K. pneumoniae* infections by analyzing plasma samples with GC × GC-TOF-MS as the first worldwide (20 CR-Kp-positive samples vs. 18 CR-Kp-negative samples). Their findings indicated that *N*-acetyl glucosamine, butanedioic acid, and myoinositol all play a significant role in CR-Kp infection. The results obtained in bacteremia studies suggest that the analysis of volatile compounds of bacterial

origin in blood/plasma may in the future constitute a tool for screening in case of suspected sepsis [67], and even identifying [66,68] and determining antimicrobial susceptibility [65]. An undoubted advantage of the analysis of gaseous samples is the possibility to confirm/exclude the presence of the pathogen in the blood immediately after taking the sample, without waiting for the culture result, enabling much faster implementation of targeted therapy, which is important in the case of blood infections.

5.3. Other bacterial infections

The analysis of bacterial volatile metabolites can also be useful in the rapid diagnosis of other infections, including those affecting the cardiovascular system (e.g., vascular graft infection and infective endocarditis) and the locomotor system (e.g., bone or prosthetic joint infections). Gòmez-Mejia et al. [10] demonstrated that direct sample analysis via SESI-MS made it possible to confirm the presence of bacterial infection within 5 min. However, to implement this method in clinical practice, it will be necessary to conduct further research and create libraries of VOCs characteristic to specific pathogens in the environment of a specific sample.

Urinary tract infections are common, with *E. coli*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Klebsiella* spp. [7] being the main causative pathogens. Although UTIs affect virtually all people, patients requiring the use of a catheter, such as ICU patients, are at particularly high risk for such infections. Roine et al. [7] analyzed 101 urine samples using the eNose detector based on IMS technology, which enabled pathogen identification at a level very similar to that of traditional bacterial cultures from urine (95% sensitivity and 96% specificity). In this respect, the IMS-based eNose detector proved to be an effective tool for identifying pathogens causing urinary tract infection in clinical practice.

Another potential application of bacterial VOC analysis is wound assessment. Wound infections are a significant healthcare problem, as the application of effective therapy can be difficult due to the formation of chronic multi-microbial infections and biofilms. In 2020, Daulton et al. [8] presented a GC-IMS-based method to control the wound for infection based only on the HS of the wound swabs. After analyzing 19 swab samples, they identified a set of 6 discriminative features for infected and colonized wounds, thus enabling their differentiation with high sensitivity (reaching 100%) and specificity (88%). Although only 3 pathogens were considered in the study (*S. aureus*, *P. aeruginosa*, and *S. marcescens*), this approach is still novel in the context of wound infection diagnosis and should be replicated using larger groups of patients.

External otitis, also known as “swimmer's ear,” is an inflammatory disease resulting from the repeated exposure of the external ear canal to water, mainly water containing *P. aeruginosa.* In 2018, Kviatkovski et al. [4] presented a newly developed device that enabled the diagnosis of *P. aeruginosa* external otitis based on the analysis of a pus sample from the ear. The proposed device contained a biosensor for 2-AAP, which has been reported as a specific VOC for this bacterium in previous *in vitro* studies [12,16,90]. The obtained results were promising, but further development is required to adapt the device for easy use in primary health care.

An innovative application of VOC analysis is for the differentiation of viral and bacterial meningitis (ME), as the latter is one of the most threatening infections of the central nervous system (CNS) [64]. In bacterial meningitis, the timely implementation of adequate antimicrobial therapy is crucial for improving the patient's prognosis; thus, the ability to identify the type of causative pathogen quickly is paramount. In 2021, Guo et al. [64] applied HS-SPME-GC-MS to analyze 58 cerebral-spinal fluid (CSF) samples: 16 from patients with confirmed bacterial ME and 42 from patients with confirmed viral ME. Their findings revealed significantly higher concentrations of ethylene dioxide and phenol in the bacterial ME samples, thus suggesting the usefulness of these VOCs in the rapid differentiation of bacterial and viral CNS in the future [64].

6. Conclusion

This paper provided a comprehensive overview of a wide range of methods for online and offline VOC analysis, along with their respective sample-preparation techniques and their advantages and disadvantages. Novel and continuously developing microextraction techniques such NTME and TFME have already proven to be effective for analyzing bacterial volatilomes in biological matrices, including saliva, sputum, BAL, blood, tissues, exhaled breath, and urine. As such, these methods are a natural choice for further research in this field. In addition, model *in vitro* experiments referring to species-characteristic and resistance-related metabolites have also contributed to significant advances in knowledge in this area over the past decade. Furthermore, a number of studies sought to investigate the factors affecting the production of bacterial VOCs and their kinetics. Despite this remarkable progress, further work is required to obtain deeper insights, especially into the interactions between bacteria species and immune cells and other areas that have gone uninvestigated thus far. The latter area is particularly important to improving the translation of *in vitro* findings to *in vivo* settings, such as disease diagnosis in humans. Another area meriting further attention is the standardization of methodology, as multiple techniques are currently employed for sample collection and preconcentration. As long as VOCs are simply adsorbed from the HS of a bacteria culture in a reproducible way over subsequent *in vitro* experiments (and are analyzed immediately), the results will be reliable. However, multiple factors can affect the findings of clinical studies, such as the conditions under which sample collection and VOC preconcentration are performed, as well as the conditions used for the transportation and storage of the sample until the final instrumental analysis. Indeed, each of these factors can significantly impact the results, as improper conditions can result in outcomes such as sample contamination or loss of the target analytes. As it is impossible to propose a single recommendation that applies to the numerous available analytical techniques, we encourage researchers to optimize and validate their analytical protocols before proceeding with clinical trials. Nevertheless, some aspects can be unified, regardless of the method used, for example: sampling the end-tidal breath (controlled by capnography) instead of mixed-expiratory air; confirming compound identification via the analysis of the respective standard; and providing a unique Chemical Abstracts Service (CAS) number when reporting substances as significant metabolites. Once such uniformity is achieved, the results from multicenter clinical trials with large patient cohorts can be compared to evaluate the true clinical relevance and cost-effectiveness of the proposed “biomarkers” of the disease in question.

CRediT author statement

Karolina Żuchowska: Writing - Original draft preparation, Reviewing and Editing, Visualization, Formal analysis; **Wojciech Filipiak:** Conceptualization, Funding acquisition, Project administration, Formal analysis, Supervision, Writing - Original draft preparation, Reviewing and Editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2023.11.005.

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