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# Present and future perspectives on mass spectrometry for clinical microbiology

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ABSTRACT

In the last decade, MALDI-TOF Mass Spectrometry (MALDI-TOF MS) has been introduced and broadly accepted by clinical laboratory laboratories throughout the world as a powerful and efficient tool for rapid microbial identification. During the MALDI-TOF MS process, microbes are identified using either intact cells or cell extracts. The process is rapid, sensitive, and economical in terms of both labor and costs involved. Whilst MALDI-TOF MS is currently the gold-standard, it suffers from several shortcomings such as lack of direct information on antibiotic resistance, poor depth of analysis and insufficient discriminatory power for the distinction of closely related bacterial species or for reliably sub-differentiating isolates to the level of clones or strains. Thus, new approaches targeting proteins and allowing a better characterization of bacterial strains are strongly needed, if possible, on a very short time scale after sample collection in the hospital. Bottom-up proteomics (BUP) is a nice alternative to MALDI-TOF MS, offering the possibility for in-depth proteome analysis. Top-down proteomics (TDP) provides the highest molecular precision in proteomics, allowing the characterization of proteins at the proteoform level. A number of studies have already demonstrated the potential of these techniques in clinical microbiology. In this review, we will discuss the current state-of-the-art of MALDI-TOF MS for the rapid microbial identification and detection of resistance to antibiotics and describe emerging approaches, including bottom-up and top-down proteomics as well as ambient MS technologies.



In the last decade, Mass Spectrometry (MS) has revolutionized the field of clinical microbiology with the introduction of Matrix-Assisted Laser Desorption Ionization - Time Of Flight (MALDI-TOF) mass spectrometry for rapid microbial identification [1]. The principle is simple. A low-resolution MS profile of intact proteins that have been ionized either from intact cells or after a simple extraction step is obtained and compared to MS profiles present in a reference library. The success of this approach is due to the fact that routine identification of pathogens can be performed in a faster, more accurate, and less expensive manner than conventional phenotypic or genotypic methods. Many hospitals worldwide are now equipped with this technology.

However, MALDI-TOF MS, which is a low-resolution approach, suffers from important limitations: For instance, some microbial species remain difficult to identify, either because they don't give a specific profile or because the database lacks the appropriate reference. In addition, the discriminatory power of MALDI-TOF MS is insufficient for reliably sub-differentiating isolates to the level of clonal complexes or strain, which would be very useful for early identification of epidemics in hospitals. Finally, virulence or resistance determinants cannot be characterized in a simple manner, which is a severe obstacle for appropriate patient care and antibiotics prescription in hospitals. Thus, new approaches targeting proteins and allowing a better identification of bacterial strains are strongly needed, if possible, on a very short time scale after sample collection in the hospital. In MALDI-TOF MS no information is obtained on the sequence and identity of the proteins that are used for the microbial identification. Therefore, an important improvement would be to identify these proteins - that are the most abundant ones - as well as less abundant ones that are the signature of a phenotype of interest (resistance, virulence). Large-scale bottom-up proteomics (BUP) approaches represent a nice alternative to MALDI-TOF MS, offering the possibility for in-depth proteome analysis. These approaches, that are based on the LC-MS/MS analysis of peptides after protein digestion, have benefited from the immense progress made in MS instrumentation and bioinformatics. Targeted proteomics is a powerful protein quantification tool

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increasingly used in systems biology, biomedical research and clinical applications. Top-down proteomics (TDP) is an emerging technology based on the analysis of intact proteins using high-resolution mass spectrometry. It provides the highest molecular precision in proteomics, allowing the characterization of proteins at the proteoform level. A number of studies have already demonstrated the potential of these techniques in clinical microbiology. In this review, we will discuss the current state-of-the-art of MALDI-TOF MS for rapid microbial identification and resistance to antibiotics and describe emerging approaches, including bottom-up and top-down proteomics as well as emerging ambient MS technologies.

## 1. MALDI-TOF MS for pathogen identification in clinics

The general MALDI-TOF MS workflow for microbial identification in hospital settings is described in Fig. 1. Although in this review we will focus on bacterial infections, the process is very similar for yeast [2] and fungi [3]. Firstly, a biological sample such as blood, urine, or swab, is collected from the patient. In these samples, the number of bacteria present at the beginning of an infection is generally too low for a direct MS analysis and several rounds of culture are required [4]. For blood samples, a broth blood culture (18–24 h) is performed to increase the yield of bacteria and to discriminate between infectious and non-infectious samples. If the blood culture is positive, a last bacterial culture step is usually performed on an agar plate to isolate the bacteria from blood cells. A bacterial colony is finally scratched from the culture plate using a pipette tip and directly deposited onto a conductive metal MALDI plate, where it is mixed with MALDI matrix. The matrix contains water, organic solvent, and strong acid that absorbs ultraviolet (UV) light. Different types of MALDI matrices are available depending on the type of sample that is to be analyzed and the optical absorption range of the laser in the source. The most frequently used matrices for proteins are 2,5-dihydroxybenoic acid (DHB), 4-hydroxycinnamic acid (CHCA or HCCA) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) [5]. After mixing, the plate is left to dry, and the matrix crystallizes. MALDI-TOF samples do not need extensive processing before analysis because the organic solvent and acid present within the matrix are usually sufficient for cell lysis and protein release. For mycobacteria (or yeast) which are enclosed in a capsule layer, commonly used MALDI matrices are not always sufficient to fully lyse the microbial cell wall. In this case, samples may undergo an extra processing step to lyse the cells, either chemically (addition of acetonitrile and formic acid), mechanical lysis or using heat [6].

Once inside the MALDI source, the matrix is irradiated using a laser beam, usually at a wavelength of 266 or 337 nm. The laser energy causes the decomposition of the crystal structure and generates a plume, or particle cloud which contains ionized bacterial proteins. MALDI ionization is a 'soft' ionization technique, meaning that labile biomolecules such as proteins can be ionized without fragmentation. After ionization, the ions are extracted by an electric field and travel towards the time-of-flight (TOF) tube

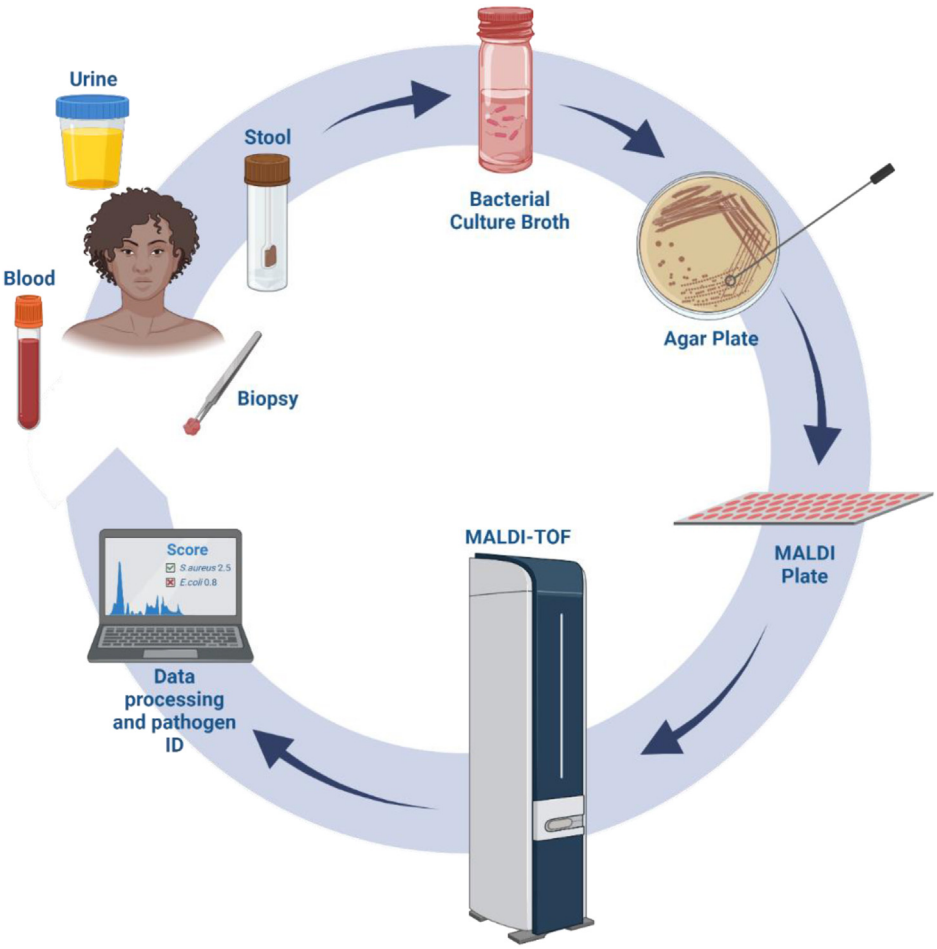


**Fig. 1.** MALDI-TOF MS workflow for microbial identification in hospital settings.

where they are separated based on their $m/z$. Some TOF analyzers are equipped with a reflector that increases the path of the ions thus increasing resolution. To simplify the analysis, the TOF analyzers used for clinical analysis have a low resolution so that all protein features (isotopic patterns) are represented by a single peak in MS spectra. At the end of the flight path, the ions hit the detector, which is usually an electron multiplier or a channel plate. A mass spectrum is generated from the ionization of intact proteins that are mainly ribosomal ones because they are small, basic and highly abundant [7]. The experimental profile obtained in the [3−20 kDa] range is then compared to a spectral library built from known pathogens and a successful identification is made if there is a spectral match (and a score is associated). The two main automated MALDI-TOF MS systems used in the clinic are the Biotyper from Bruker [8] and Vitek MS from Biomérieux [9] that both include proprietary libraries and software tools. Both instruments are FDA approved.

The use of MALDI-TOF MS for the identification of microorganisms covers many types of bacteria, as well as fungi and yeast [10]. The method has a high success rate and can generally identify bacteria at the species level in about 90 % of the cases. It works for both Gram-positive and Gram-negative bacteria in a faster manner than biochemical techniques [11], which improves patient care in hospital settings. However, although MALDI-TOF MS is an effective technique for microbial identification, it does not provide direct information on bacterial resistance to antibiotics, which is now a major public health problem. Specific MALDI-TOF MS methods have thus been developed to this aim.

## 2. MALDI-TOF MS and antibiotic resistance

Drug-resistant bacterial strains are becoming widespread in hospitals, seriously compromising patient care. Typically, MALDI-TOF MS is used to provide bacterial identification, and an antibiotic susceptibility test is then required to determine the resistance status of the microbial pathogen. A fast microbial antimicrobial sensitivity test (AST) that would encompass as many types of resistance as possible is strongly needed.

Carbapenems are a widely used class of broad-spectrum antibiotics, and many bacterial strains are showing resistance to this class of drugs. The identification of carbapenemase-associated proteins by MALDI-TOF MS has been demonstrated for several bacterial pathogens including *Acinetobacter baumannii*. For this pathogen, protein biomarkers could be detected from 51 resistant strains with a sensitivity of 96 % and a specificity of 73 % in comparison to the classical Minimum Inhibitory Concentration (MIC) determination test [12]. Figuera-Espinosa et al. [13] have developed a MALDI-TOF based procedure to detect the presence of Class A carbapenemase KCP-2 directly from positive blood cultures. Antibiotic resistance was confirmed by a single diagnostic peak corresponding to a biomarker which was consistently found in KCP-2 positive samples and absent in controls. This technique showed 100 % accuracy for the detection of KCP-2.

The MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay (MBT-ASTRA) is an antimicrobial resistance assay, that can detect general drug-resistance in a wide range of bacterial pathogens, without the need for specific optimization or species or drug-specific assay development [14]. This technique is based on a software that compares the area under the curve (i.e. the bacterial biomass) from MALDI-TOF MS spectra achieved from bacteria that have been exposed to antibiotic drugs. Resistant bacteria will have a larger area under the curve (larger biomass) compared to susceptible ones. Theoretically, this assay, which takes about 4 h, could be used across multiple bacterial species and strains, against all classes of antibiotics tested the method for the identification of amoxicillin and cefotaxime-resistant *Escherichia coli* from positive bacterial cultures [15]. In a study by Sauget et al. [16], the MBT-ASTRA produced the same result as the reference method for 97 % of amoxicillin-treated samples and 83 % of cefotaxime-treated samples. Although the assay may be better suited to some antibiotics than others, it offers a simple technique for the global identification of antibiotic resistance for a wide variety of bacteria.

In summary, several methods based on MALDI-TOF MS have been developed for drug-resistance screening for various pathogens and antibiotics. However, there is currently no generic method providing the requested information in a simple way and in a short period of time. An important drawback of MALDI-TOF MS is that it is associated with a limited depth of analysis, in part due to the lack of analyte separation (by chromatographic techniques) prior to MS. Moreover, it does not provide information on the identity of the proteins that lead to the experimental profiles used for the bacterial identification. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is now routinely used for in-depth proteome characterization, allowing the identification and quantification of thousands of proteins from various types of biological samples. These proteomics analyses can be performed at the peptide (bottom-up) or protein (top-down) level and represent a nice alternative to MALDI-TOF MS to deeply characterize bacterial pathogens in a clinical context, including virulence and resistance determinants.

## 3. Going beyond MALDI-TOF MS with bottom-up proteomics

In bottom-up proteomics (BUP), information about the proteins is inferred from the peptide analysis, building the protein characterization from the 'bottom-up'. Proteins are digested into peptides using an enzyme such as trypsin, and the peptides are then separated by LC and analyzed by MS. Peptide identification is achieved by matching the tandem mass spectra (MS/MS) generated from peptide fragmentation with theoretical tandem mass spectra generated from *in silico* digestion and fragmentation of a protein database [17].

BUP techniques can be used to analyze proteins in a targeted or untargeted manner. Untargeted analysis allows a snapshot of the bacterial proteome, where thousands of proteins can be identified with a high degree of confidence. This is useful for observing general differences between bacterial strains and characterizing bacterial strains by measuring their proteomic 'fingerprint'. Targeted approaches can be used for the analysis of chosen proteins of interest. This approach focuses on the identification of a subset of proteins, so fewer proteins are identified overall, but those that are identified are those of biological relevance. Quantification of bacterial proteins is possible using both targeted and untargeted approaches.

Data-dependent acquisition (DDA), or shotgun proteomics, is the method of choice for many laboratories. DDA is an untargeted approach where the most abundant precursors are selected for MS/MS fragmentation, and the spectra are compared against a database for protein identification.

Fleurbaaji et al. [18] used LC-MS/MS in DDA mode to identify extended-spectrum beta-lactamases (ESBLs) from positive blood cultures. ESBLs are enzymes which confer drug resistance to cephalosporins, penicillins, and beta-lactam antibiotics, among others. It is one of the most common forms of antibiotic resistance in Gram-negative bacteria. They were able to identify 400−800 bacterial proteins per blood culture with beta-lactamase in the top 10 % of proteins. The test did not identify beta-lactamase from any of the ESBL-negative blood cultures.

Targeted methods such as Selected Reaction Monitoring (SRM) [19] or Multiple Reaction Monitoring (MRM), and more recently

Parallel Reaction Monitoring (PRM) [20] have been proposed as efficient strategies for deep microorganism characterization [21–23]. SRM experiments are often performed on triple quadrupole mass spectrometers where precursor ions are selected in the first quadrupole and fragmented in the second quadrupole. Target-specific fragment ions are then selected in the third quadrupole for detection. Typically, several fragment ions are successively monitored. PRM experiments are performed on systems able to record whole fragment spectra such as Quadrupole-Orbitrap mass spectrometers. In PRM, all fragment ions of a selected precursor are measured in parallel. The selection of the suitable target peptides and fragment ions as surrogates for the target proteins is essential to the sensitivity, specificity, and analytical power of targeted assays.

*Staphylococcus aureus* is a common bacterium which is frequently responsible for hospital-contracted infections. Methicillin-Resistant strains (MRSA) are particularly problematic in hospital settings. MRSA produce a wide array of resistance mechanisms, characterized by the presence of penicillin binding proteins such as PBP2a and PBP2c. Several virulence factors such as the syndrome toxin 1 (TSST-1) and Panton-Valentine Leukocidin (PVL) toxins can also be produced. Charretier et al. [21] developed an SRM approach that provides bacterial identification, virulence assessment, methicillin resistance as well as epidemiological typing in *S. aureus* sepsis patients. The method is fast (60–80 min) and can be performed on positive blood cultures or bacterial colonies.

Another BUP approach, which is emerging for the analysis for very complex mixtures, is Data-independent acquisition (DIA). In DIA experiments, all peptides in pre-defined *m/z* windows are fragmented together, which is useful to extract information from lower abundance ones. DIA can therefore overcome some of the issues with reproducibility that are associated with DDA. Compared to DDA, DIA approaches provide higher accuracy, sensitivity, and throughput and is therefore being used increasingly often in pre-clinical and clinical analysis.

DDA and DIA modes were combined in a study by Roux-Dalvai et al. [24] In a first training step, libraries of peptides are obtained on pure bacterial colonies in DDA mode, their detection in urine was then verified in DIA mode, followed by the use of machine learning to define a peptide signature which can be used to distinguish each bacterial species from the others. Using this method, the authors were able to identify bacterial species in urine in under 4 h and it was tested for the 15 species of bacteria which are responsible for 84 % of urinary tract infections. The machine learning model was able to produce 82 peptide signatures from 31,000 peptides which were measured from 190 samples. In this work, DIA combined with machine learning was effective for furthering the capabilities of classical DDA experiments and provides an example of the value of DIA analysis for clinical applications.

These examples show that BUP techniques can represent an interesting alternative to MALDI-TOF MS approaches, allowing the identification of bacterial strains, and the characterization of potential resistance and virulence factors in a single run [21]. However, BUP approaches are unsuitable to achieve information at the proteoform level. The term 'Proteoform' designates all of the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and post-translational modifications [25]. Studying bacterial strains at the proteoform level is important since it can reveal a deeper cohort of biomarkers which are proteoform specific and cannot be distinguished by BUP techniques. To characterize proteoforms, protein samples need to be analyzed using top-down proteomics (TDP) approaches, which are based on high resolution mass spectrometers such as Orbitrap type ones [26].

## 4. Going deeper with top-down proteomics

In TDP, proteins are analyzed intact by LC-MS/MS without prior enzymatic digestion, as is used in BUP. Compared to MALDI-TOF MS, where proteins are also kept intact, TDP can be used to increase the depth and precision of the analysis by the addition of (i) an LC step that separates proteoforms before their introduction in the mass spectrometer (ii) a fragmentation step leading to MS/MS data that can be used for proteoform identification through database search (with dedicated software tools). Therefore, although proteoforms differing by a single amino acid can be very difficult to discriminate by BUP (as only partial sequences are obtained), they will be much more easily characterized in TDP. In addition, by keeping the proteins intact, the sample complexity is largely reduced compared to BUP. It is also worth noting that bacterial genomes are not extremely complex and bacterial proteins are rather small (<30 kDa), which make them ideal targets for TDP experiments. TDP workflows are still underdeveloped compared to their BUP counterparts due to challenges in all steps of the analysis: sample preparation [27], online proteoform separation, efficient fragmentation for large proteoforms (>30 kDa) and data analysis with a lack of robust and efficient bioinformatics and statistic tools [28].

The ability to obtain a highly detailed, untargeted snapshot of a bacterial infection is important because bacteria can replicate in the host quickly, and specific proteoforms generated from the addition of Post Translational Modifications (PTMs), alternative splicing or single nucleotide polymorphisms (SNPs) can affect the expression or virulence of the bacteria. This not only has grave implications for a patient in hospital suffering from a bacterial infection, but also for screening food and pharmaceuticals.

A comprehensive understanding of bacterial proteoforms is a major leap forward for the characterization of different infection pathologies. C. Ansong et al. [29] used a high-resolution mass spectrometer (Orbitrap Velos) to measure 1665 proteoforms arising from *Salmonella typhimurium*. Several of these proteoforms were previously unknown. The authors demonstrated that under infection-like conditions, there is a strong induction of cysteine biosynthesis in the Gram-negative bacteria leading to S-cysteinylated proteoforms, that had not previously been observed using BUP approaches. For *Neisseria meningitidis*, the causative pathogen in cerebrospinal meningitis, proteoforms of the PilE protein carrying phosphoglycerol groups could tightly been associated with crossing of the epithelial barrier and access to the blood stream [30]. PilE is the major component of Type IV pili, which are filamentous organelles protruding from the bacterial membrane and major virulence factors for many Gram-negative pathogens. For the same protein, highly glycosylated proteoforms were observed in meningitis patients and linked to immune escape [31]. Another study determined that rare O-mycoloylations on several proteins were critical for their localization to the outer membrane of *C. glutamicum* [32]. In a more recent work, TDP was applied to detect the PBP2a resistance protein from *S. aureus* clinical isolates using a 5-min liquid chromatographic separation [33]. Finally, high-resolution TDP LC-MS/MS methods have already been used in several pre-clinical studies to discriminate between similar strains of bacteria with a high degree of accuracy (Fig. 2).

*Salmonella enterica serovar Typhimurium* and *S. enterica serovar Heidelberg* are two of the top ten serovars responsible for *Salmonella* poisoning from food and present highly similar amino acid sequences. Although it is possible to distinguish between the two

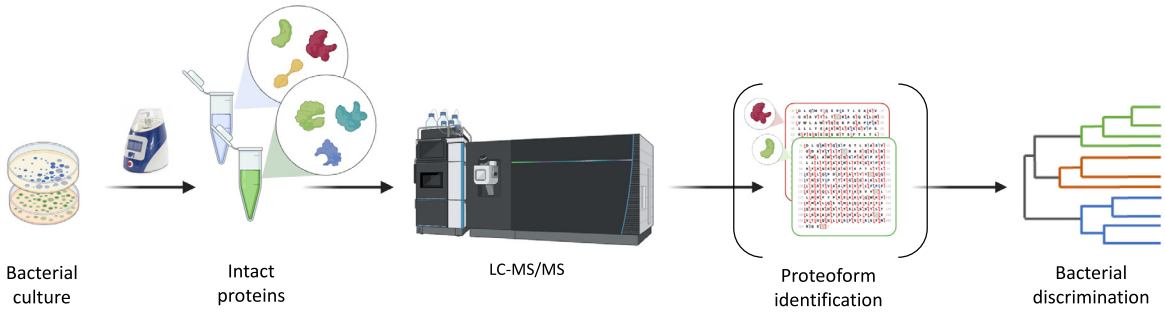


**Fig. 2.** Top-Down Proteomics pipeline for the analysis of bacterial pathogens at the proteoform level.

strains using PCR assays, it is a lengthy process requiring multiple PCR targets and a validated standard. TDP is the ideal tool to measure differences between strains because mass shifts can quickly be interpreted as the presence of PTMs or SNPs, whilst unmodified proteins or proteoforms are highly conserved between strains and are therefore unlikely to change in mass. Using TDP, McFarland et al. [34] were able to identify and distinguish between *S. Typhimurium* and *S. Heidelberg*, where the differences between the two strains are limited to six SNPs, which produce a very small mass shift compared with the non-modified protein. Using TDP and electron-based fragmentation, Dupré et al. [35] optimized a top-down proteomics workflow for the identification and discrimination of different bacterial species. *E. coli* K12 lysate was used for the initial optimization, starting with sample preparation and then LC conditions such as the use of different columns for the chromatographic separation of the proteins were tested. Finally, MS conditions were optimized, and thirty-nine LC-MS/MS conditions were compared. Using these methods, the authors were able to identify more than 3500 proteoforms from 12 endobacterial species allowing their differentiation. This work demonstrates that high-resolution TDP experiments can be used to discriminate between closely related bacterial species at the proteoform level, but the database search was challenging for less-well studied bacterial species. A software tool named diagnoTOP [36] was further developed to achieve the discrimination bacterial strains but without any database search, which is a major step forward. Note that, as in BUP experiments for proteins, the usual way to characterize proteoforms is to perform a database search using a dedicated software tool. An established and commercial software in TDP is Prosight [37], but several others are also available [38]. A recent comparison of TDP software shows that each algorithm can produce different outputs from the same data, so users should be aware that TDP analysis pipelines are still not standardized [39].

## 5. Other emerging MS-based methodologies

LC-MS/MS techniques require some level of sample preparation, such as protein extraction, purification, or sample cleanup (to remove salts and detergents which are not compatible with MS analysis) before the LC separation step. These pre-processing procedures require time, resources, and manual input. However, with the use of ambient MS methods such as desorption electrospray ionization (DESI) and Liquid extraction surface analysis (LESA), samples can be analyzed directly without any prior processing steps and MS analysis can be carried out in real time.

DESI-MS works by directing charged electrospray droplets directly onto a surface containing the analyte. Unlike electrospray ionization (ESI), which requires purified samples in a liquid form, DESI — and other ambient MS techniques - can be used for raw samples, such as bacterial colonies grown on medium in a Petri dish. Charged droplets are formed and when they collide with the surface containing the analyte, the collision forms ions which can then be directed towards the MS. As the samples do not need to be introduced into the vacuum prior to MS analysis, the study of samples *in vivo* such as bacterial colonies is possible using ambient methods. A study by Song et al. [40] used DESI-MS to identify various microorganisms directly from biofilm growing on agar, and to characterize the antibiotics produced by the bacteria *in vivo*. In the study the authors used *Bacillus subtilis*, a Gram-positive bacterium which produces several antibiotics which are of interest to clinicians. They were able to acquire high-quality MS spectra, predominantly identifying lipopeptides secreted by the bacteria. The analysis shows that lipopeptides are readily available from growth medium, and well suited for DESI experiments because they are powerful surfactants, and therefore reduce the surface tension of water allowing better ionization by DESI. They also have high molecular weights, making them easy to distinguish from the signals which originate from the growth medium.

LESA-MS is a technique that can also be used with TDP for the analysis of live bacterial cultures. LESA is an ambient ionization technique where the analyte is mixed with a droplet of solvent and then passed through a nano electrospray ionization chip (nanoESI) [41]. The droplet is deposited directly onto the sample via a pipette tip and is used to create a micro junction between the pipette tip and the sample. After ionization, the drop is re-aspirated, and the analyte is introduced to the MS via an electrospray capillary. LESA-MS can be applied to a wide variety of samples which are of interest in a clinical setting, such as blood, tissue sections and living bacterial colonies on agar [42]. When tested against Gram-positive and Gram-negative bacterial species, 40 proteins were identified for each one. 16 of those were proteins that had only been described by genome sequencing and one was a novel protein. LESA was used to distinguish between several different bacterial species including *E. coli, Pseudomonas aeruginosa, S. aureus, S. pneumoniae, S. oralis and S. gordonii. De novo* sequencing was also possible for an unknown protein from an unknown strain of *S. aureus*. Havlikova et al. [43] used LESA-MS to identify three pathogens which are frequently responsible for infections acquired in hospital (ESKAPE pathogens: *Klebsiella pneumoniae*, *P. aeruginosa* and two strains of *S. aureus*). They were able to identify 24 proteins from 37 MS/MS spectra, showing that LESA-MS is a technique that works for both Gram-positive and Gram-negative bacteria.

## 6. Conclusion and perspectives

MALDI-TOF MS is the current gold-standard in clinics because it provides a reproducible and accurate identification of bacterial pathogens from patient samples such as blood and urine at low cost

and with high throughput. However, it suffers from several drawbacks such as low resolution, which means that it is difficult to distinguish bacteria beyond the species level and is unsuitable for the discrimination of closely related ones. Data analysis also relies on pre-existing spectral libraries which complicates the analysis of unknown pathogens. Other MS techniques are now being developed in pre-clinical and clinical studies to address these shortcomings. Bottom-up proteomics can provide a high-resolution snapshot of bacterial proteins that can be used to identify bacterial species and potential antibiotic resistance. Targeted methods have been proposed for in-depth characterization of microorganisms in multiplexed analysis, allowing microbial identification (I), antibiotic-resistance detection (R), virulence assessment (V) and epidemiological typing information (T) to be achieved in less then 80 min from the analysis of positive blood cultures of *S. aureus* sepsis patients. Top-down approaches, although less sensitive, bring an unrivalled degree of precision with the characterization of proteins at the proteoform level, providing a deeper and more specific cohort of unique biomarkers. New experimental techniques such as DESI and LESA allow bacterial analysis directly from patient samples and even from patient tissue or bacterial culture on agar with no pre-processing steps, drastically shortening the analysis time from sample collection to diagnosis. Whilst many of these techniques are still undergoing development, it can be expected that the future of MS in microbiology will include a wide variety of MS-based techniques and methods optimized for the analysis of bacteria directly from patient samples and biofluids, with fast processing time and automated MS analysis that is capable of characterizing new bacterial species without reliance on existing spectral databases. These techniques combined with machine learning and advanced MS data processing technology represent a promising future for clinical microbiology.

## Declaration of competing interest

None.

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