Review

Proteomics boosts translational and clinical microbiology☆☆

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ABSTRACT

The application of proteomics to translational and clinical microbiology is one of the most advanced frontiers in the management and control of infectious diseases and in the understanding of complex microbial systems within human fluids and districts. This new approach aims at providing, by dedicated bioinformatic pipelines, a thorough description of pathogen proteomes and their interactions within the context of human host ecosystems, revolutionizing the vision of infectious diseases in biomedicine and approaching new viewpoints in both diagnostic and clinical management of the patient.

Indeed, in the last few years, many laboratories have matured a series of advanced proteomic applications, aiming at providing individual proteome charts of pathogens, with respect to their morph and/or cell life stages, antimicrobial or antymycotic resistance profiling, epidemiological dispersion. Herein, we aim at reviewing the current state-of-the-art on proteomic protocols designed and set-up for translational and diagnostic microbialological purposes, from axenic pathogen’s characterization to microbiota ecosystems’ full description. The final goal is to describe applications of the most common MALDI-TOF MS platforms to advanced diagnostic issues related to emerging infections, increasing of fastidious bacteria, and generation of patient-tailored phylogenotypes.

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1. Introduction

In the last few years, proteomics has dealt with emerging or re-emerging pathogens, including neglected agents, in order to provide support in taxonomic microbial classification, invasion mechanisms, life cycle stage switching, antigen variation, drug target and drug resistance discovery, and infection epidemiology.

Genomic data are currently growing at an extraordinary rate. However, this huge amount of descriptive work has only scraped the surface of the microbial biodiversity, endowed with environmental, opportunistic and pathogenic strain patterns, still lacking comprehensive functional insights into the microbe-host cross-talk. The highly microbial diversity indicates strategies devised to potentiate specialized adaptation mechanisms within host or ecological niches, through genetic material transfers, post-transcriptional machineries, proteome modulation and controlling in response to specific external stimuli. While genomics may provide a comprehensive snapshot on everything related to nucleic acids, proteomics ensures the actual description of specific proteins or complexes of proteins in their biological milieu. Furthermore, proteomes are much more multiform and dynamic than related up-stream genomes, hence requiring complex experimental pipelines, including separation, detection, and data processing, to highlight either protein content and/or modifications on them. The complexity item can be managed or off-line mode coupled-tandem MS (i.e., shotgun proteomics).

The successful coupling of multidimensional separations with mass spectrometry (MS) for protein and peptide analyses has been achieved with the advent of the ionization Matrix-assisted laser desorption ionization (MALDI) and Electrospray ionization (ESI) techniques, assisted by the evolution of new powerful MS instrumentations. While MALDI is usually combined with gel-based separations, ESI is frequently coupled with “on-line” High-pressure liquid chromatography (HPLC) or reversed-phased (RP) LC separations.

Two main analytical approaches are commonly used for protein analyses for proteome separation and characterization: “top-down” and “bottom-up”. The top-down is an emerging design for the analysis of intact proteins separated by HPLC and identified by MS. In the bottom-up approach, samples require a gel-based purification step, followed by an enzymatic or chemical digestion that provides peptides separated by multidimensional chromatography, such as 2-D-HPLC, and finally identified by tandem MS. Otherwise, the crude protein extract may be digested directly, followed by on-line LC or off-line mode coupled-tandem MS (i.e., shotgun proteomics).

Peptide and intact protein analysis can be conducted using either the time of flight (TOF) MS, or the Fourier transform ion cyclotron resonance (FT-ICR), that ensures a performance in a wide mass range and, in the case of FT-ICR, a high mass accuracy. Other types of instruments (e.g., hybrid linear ion trap [LTQ]-orbitraps, LTQ-FTICRs, or quadrupole ion trap) can also be applied to identify proteins. From a general point of view, MALDI-TOF MS is ideal for relatively clean and less complex samples, with the targeted protein resulting in the starting sample. Infusion ESI-MS/MS should be used when the sample is clean and at low complexity, with the scope of characterizing all multiple analytes and possible modifications on them. The complexity item can be managed in terms of appropriate proteomic technological platforms by including in translational and diagnostic microbiology a miscellaneous of methodological pipelines to analyze global proteomes or part of them. This will therefore reflect microorganism status and complexity to respond to specific needs: i) proteomic phenotyping supported by MALDI-TOF MS peptide fingerprinting; ii) proteome charting by bottom-up shotgun profiling; iii) sub-proteome relative and absolute quantitation by differential labelling and targeted analysis, respectively; and iv) community-based analysis of operational taxonomic units (OTUs) from microbial communities (e.g., gut, airway, salivary microbiota) by LC-ion trap MS/MS. Therefore, dealing with pathogens or opportunistic pathogens by proteomic approaches means to design and set-up the proper technology for general microbiological or specific diagnostic purposes. Indeed, each diagnostic or translational task needs specific pre- and analytical solutions and proper technological platforms. Therefore, a wide range...
of proteomic platforms today ensures both bio-detection and comprehensive description of protein patterns and protein markers, through whole proteome mining of microbial pathogens [13]. Against pursuing with the characterization of axenic microbes, which represents a shortcut in pathogen characterization, major challenges still rely on the optimization of ID procedures for fastidious, slow growing and anaerobe bacteria and in the generation of microbial charts describing the complexity of polymicrobial cultures from direct samples (e.g., blood cultures, liquor, urine, bronchoalveolar lavage [BAL]) or microbial ecosystems (Fig. 3). However, the pathogen is now considered in the complexity of its ecological niche within the host (infection site) or in the context of its relationships with environment and host. For these reasons, the systems biology may represent the choice of election in the study of microbial pathogens integrated in their ecological “context” (e.g., “gut microbiota”) [14]. MS and MS/MS proteomic approaches are determinant for all pathogens characterized by complexity of stage or morph (type) (e.g., filamentous fungi) and multiple components of the life cycle (e.g., oral–faecal parasites, such as Cryptosporidium spp., a metabolic evolutionary model for many parasites, fungal and anaerobe or modified aerobe bacteria). Nowadays, one of the most revolutionary proteomic innovation lies in the direct and robust top-down MALDI-TOF MS, which represents a peerless tool for microbiological diagnostics, becoming a reference method to identify bacteria, yeasts and filamentous fungi at genus, species and strain level [15].

Fig. 1 – Main proteomic approaches driving discovery and characterization of peptides and proteins of translational and clinical interest in microbiology. The different objectives require specific approaches to solve the diverse translational and diagnostic needs.

Fig. 2 – Proteomic technological platforms and related applications to current translational and clinical microbiology. The increasing complexity of the presently employed proteomic approaches is related to a growing technological complexity.

Fig. 3 – Main fields of proteomic applications to basic (axenic cultures) and advanced (direct samples, bacterial communities) microbiological pipelines.
A standardized and simple protocol, based on single plate colony picking and direct streaking onto MALDI polished target plate, easy chemical treatment (e.g., peptide extraction by formic acid, FA), and proton transfer by α-cyano-4-hydroxycinnamic acid (CHCA), allows prompt and accurate microorganism IDs, generating peptide-based spectra profiles interpreted by original and customized databases (Fig. 4). The generation of peptide profiling makes the top-down proteomics as one of the most efficacious and advanced strategy in diagnostic microbiology pipelines.

2. MALDI-TOF MS-based ID in clinical microbiology

Pathogen characterization, based on MALDI-TOF MS fingerprinting profiling, now represents an affordable and consolidated procedure in routine diagnostic microbiology which is progressively covering the entire spectrum of the four-step diagnostic pipeline paradigm: detection, ID, susceptibility testing, epidemiology. Wide microorganism profiling multiplicity, available as original reference database (ORD, provided with the BioTyper and Vitek MS softwares) [16], is currently guaranteeing fulfilment of investigation for the most important and common pathogens (Fig. 4). If a clinically relevant pathogen is detected by solid medium growth, it can be further tested for ID, drug susceptibility, and epidemiological typing, independently or after growth condition setting. The MALDI-TOF MS protein fingerprint employs a pattern-matching algorithm to compare microbial cell extracted peak lists with a reference database, hence creating a consensus “biomarkers” peak list, characterized by averaged m/z and intensity. However, only a couple of works on biomarkers’ selection has provided the first proof of evidence for bacteria and fungi classifiers associated with ID MS profiling [17,18]. The Bruker MALDI BioTyper is a MALDI-TOF MS based bench-top platform for rapidly identifying bacteria, yeasts and filamentous fungi. Its wisely curated database includes quality-controlled entries of more than 3900 strains from over 2000 well-characterized microbial species (http://ir.bruker.com/phoenix.zhtml?c=121496&p=irol-newsArticle&ID=1552077&highlight=) [19]. However, the dedicated design and compilation of customized new reference databases (NRDe) is often crucial to “resolve” and assess specific fingerprinting profiling not properly matching against ORDs. Indeed, while MALDI-TOF MS misidentification rate appears extraordinarily low, as showed by a plethora of works published so far [17,20–25], the bottleneck of the ID pipeline still lies in the inadequacy of reference libraries, lacking “adequate” matching patterns for “each type” of bacterial species and/or strain. Database upgrades are therefore essential elements to refine the MALDI-TOF MS technique, allowing the method to increase its discriminatory power.

Fig. 4 – Pattern multiplicity of MALDI-TOF MS fingerprinting of microorganisms species and types. The generated “profiles” are interpreted by matching them against reference spectra databases, hence assigning each of them to a specific pattern and providing bacterial identification.
The reservoir of fastidious bacteria [26], such as Legionella spp. [27–29], Haemophilus influenzae [30], Helicobacter pylori [31], Bartonella spp. [32], Mycobacterium spp. [33–36]; anaerobes [37–41]; Gram-negative and Gram-positive bacteria of clinical relevance [42–51] and aero-tolerants [52] offer the major challenging competition amongst clinical microbiological laboratories for advanced database compilation and easy implementation of routine ID. However, the need to identify bacteria at biotype and serotype levels (e.g., for Brucella spp. and Shiga toxin-producing Escherichia coli, STEC) [53–58] and the emerging interest in environmental [29,59,60], food-related [61–67] and bioterrorism-agent bacteria [68,69], is definitely improving the MALDI-TOF MS-based routine in clinical laboratories [26]. Microorganism ecology and metabolism (e.g., environmental or clinical yeast isolates, filamentous fungi or morphs, fastidious bacteria), have also driven until now pre- and analytical process setting for MALDI-TOF MS optimization, responding to specific patient-tailored clinical issues (e.g., clinical categories such as immunocompromised or chronic patients and major related infections) [70–74], sample types (e.g., culture-based or direct sample) [75,76], and infection spreading modality (e.g., nosocomial bacterial or fungal strains) [18,77] (Fig. 3).

In the basic procedure, starting from a cultured colony, ID is performed by matching the measured peptide mass fingerprinting against the MALDI BioTyper ORD, which can be followed by a final review performed by a trained microbiologist. Therefore, in the basic workflow, ID can be carried out within a few minutes including database searching. Multiple colonies can be spotted on a single target, and simultaneous 30–50 IDs can be approximately performed every hour. At basic-level (i.e., microbial ID), the MALDI BioTyper up to now seems to offer high specificity, almost no false positive rates, little operational costs, low technical barriers for operators, and considerably faster time-to-result compared to classical morphotyping (e.g., microscopic and macroscopic ID), phenotyping (e.g., biochemical assimilation patterns) or advanced genotyping methods (e.g., rep-PCR, sequencing). However, customized library compilation may dramatically improve specific diagnostic pipelines, such as multi-drug resistant (MDR) strain tracking, specific pathogen lineages IDs (e.g., nosocomial circulating strains from immunocompromised patients, children or neonates; food-related strains). Nevertheless, the crucial drawback of private/local databases lies in their limited availability to the scientific community, which actually hampers the sharing of accessible MS profiles for inter-laboratory consultation. Recently, Böhme et al. in their paper [78], have thoroughly described the web-based SPECLUST application (http://bioinfo.thep.lu.se/speclust.html), able to compare peak mass lists, and the Spectra-Bank database (www.spectrabank.org), a reference repository for both microbial ID and “typing”. Indeed, such tools may provide phylotypearchic clustering topologies, similarly to previous tracing methods for genotyping, extending the concept of “gene ontologies” to phenotype (proteome) ontologies [79]. Stable phenotype profilings strongly correlate with pre-analytical procedures. Indeed, engineering of reference databases relies on unvarying growth conditions. Especially for filamentous fungi, standardised growth conditions on selected solid media, represent the key element to produce technical reference and analytical profiling from heterogeneous life cycle stages, hence providing reproducible proteomic profiling and related reference databases [18,80]. On the other side, also bacterial proteins are affected by culture conditions, with different proteins produced by vegetative bacteria when they are cultured in different growth media [81]. It seems that a core set of small proteins remains constant under different culture media conditions, including minimal medium (i.e., M9), rich media (i.e., tryptic soy or Luria-Bertani broths) and blood agar plates [82]. Sample enrichment is another essential factor increasing MALDI-TOF MS performance [23]. For the ID of a significant proportion of yeasts and filamentous fungi, an extraction method prior to the analysis in the mass spectrometer is recommended to obtain appropriate spectra. Due to the low marginal costs and the extreme speed, MALDI-TOF MS can indeed improve laboratory efficiency when used early in ID protocols. However, lengthier, more labour-intensive, and costlier techniques can be reserved for the minority of isolates not detected with high confidence by MALDI-TOF MS.

MALDI-TOF MS also has the potential to identify pathogens in direct samples, such as urine and blood cultures. Furthermore, the potential for ID at the serotype or strain level, and antibiotic resistance profiling within minutes make MALDI-TOF MS an ongoing revolution in the clinical microbiology laboratory. However, despite replacing phenotypic IDs of bacteria, there is still no consensus standard regarding requirements for MALDI-TOF MS validation prior to clinical use in the United States [83]. Indeed, density of organism spotting onto a steel target, direct overlay of bacteria with CHCA, and microbiological variables, such as culture medium, growth temperature, serial subcultures, and modification of ID acceptance criteria at species level, may be considered critical issues for technical validation. Application of optimized BioTyper score cutoffs for species-level IDs, increased ID rate by 6.75% for the enteric Gram-negative bacteria and 4.25% for the non-fermenting Gram-negative bacteria, compared to the manufacturer’s recommended score cutoff [83]. Indeed, MALDI-TOF MS, using the unamended database supplied with the system BioTyper, still suffers in identifying bacteria commonly isolated in cystic fibrosis (CF) patients, such as mucoid Pseudomonas aeruginosa, when direct colony technique and routinely extraction procedure are performed, compared to phenotyping methods (e.g., culture, API systems) [81]. However, in the paper by Desai et al. [72] a set of 29 Burkholderia cepacia complex isolates, were identified by MALDI-TOF MS for 85% of the isolates, compared to only 34% with the conventional methods. Another study [84] recently focused on pre-analytical steps to identify Gram-positive cocci, considering three sample preparation methods: the “direct transfer”, the “direct transfer-FA method” and the “ethanol-FA extraction”. The rate of correct IDs at genus level was approximately 99% for all three sample preparation methods. The species ID rate was significantly higher for the “direct transfer-FA method” and the “ethanol-FA extraction” (both 77.6%), compared to the “direct transfer” (64.1%). In a following prospective study, 1619 clinical isolates of Gram-positive cocci were analysed under routine conditions, using the “direct
transfer-FA” preparation, and by conventional biochemical methods. For 95.6% of the isolates a correspondence between conventional and MALDI-TOF MS ID was observed. Two major limitations were encountered using MALDI-TOF MS: the differentiation of Streptococcus mitis group members and the Streptococcus dysgalactiae ID. However, the Bruker MALDI BioTyper, using the “direct transfer-FA” sample preparation method, appeared highly reliable for Gram-positive cocci ID, suggesting an alternative algorithm in clinical laboratory [84]. Another paper [85] has recently considered the ID of 239 aerobic Gram-positive isolates, evaluating four direct smear methods, including heavy (H) and light smears (L), with and without a direct FA overlay and assigning as “quality measure” a numerical value. The authors found that H + FA produced optimal MALDI-TOF MS ID scores and the highest percentage of correctly identified bacteria. Using a score of ≥2.0, 183/239 isolates (76.6%) were identified at genus-level and out of the 181 isolates resolved at species-level, 141 (77.9%) were correctly identified. To maximize the number of correct IDs, minimizing mis-IDs, the data were analysed using a score of ≥1.7 for genus- and species-level ID. Using this score, 220/239 isolates (92.1%) were identified at genus-level and of the 181 isolates resolved to species, 167 (92.2%) presented accurate species ID. A subset of pre-analytic factors influencing MALDI-TOF MS ID was also considered. Frequent subcultures increased the number of unidentified isolates, while incubation temperature and media subcultures did not alter ID rate [84]. Microorganism IDs directly from positive blood culture represent a major step forward to reduce the ID turnaround time (TAT) and may lead to earlier appropriate treatment of bacteremia. Indeed, in the paper by Vlek et al. [86], authors demonstrated the role of direct MALDI-TOF MS, routinely performed on positive blood cultures. In total, 253 episodes of bacteremia were included in the study, of which 89 approached by MALDI-TOF MS-based diagnosis and 164 without MALDI-TOF MS usage (e.g., control period). Remarkably, the direct performance of MALDI-TOF MS on positive blood cultures reduced the species ID time by 28.8 h and was associated with an 11.3% increase in the number of patients receiving appropriate antibiotic treatment 24 h after positivity (64.0% without MALDI-TOF MS versus 75.3% with MALDI-TOF MS usage) [84]. Also in the diagnosis of urinary tract infections (UTIs), ID of the urinary tract pathogens (automated screening, plate cultures, and biochemical characteristics-based IDs) may be implemented by MALDI-TOF MS. With this purpose, in the paper by Ferreira et al. [53], 260 urine samples, detected as positive by the flow cytometry screening device (UF-1000i), were

Fig. 5 – MALDI-TOF MS patterns of ampicillin and ertapenem resistance for Escherichia coli and Klebsiella pneumoniae strains (panel A and B, respectively). Panel 1A: MALDI-TOF MS spectrum of ampicillin; panel 2A: MALDI-TOF MS spectrum of ampicillin after incubation with a β-lactamase-producing (ESBL+) E. coli strain; panel 3A: MALDI-TOF MS spectrum of an ESBL- E. coli strain. Panel 1B: MALDI-TOF MS spectrum of ertapenem; panel 2B: MALDI-TOF MS spectrum of ertapenem after incubation with a carbapenemase-producing (KPC+) K. pneumoniae strain; panel 3B: MALDI-TOF MS spectrum of a carbapenemase-negative (KPC-) K. pneumoniae strain.
processed by culture and MALDI-TOF MS. Twenty samples were positive in the screening device but negative in culture, consistently with MALDI-TOF MS negativity. Two-hundred and thirty-five samples displayed significant growth of a single morphological type in culture, and 220 of them showed bacterial growth of >10^5 CFU/ml. Microorganism IDs in this group were almost coincident at the species level in 202 cases (91.8%) and at the genus level in 204 cases (92.7%). The most frequent microorganism was *E. coli* (173 isolates). MALDI-TOF MS identified this microorganism directly from the urine sample in 163 cases (94.2%) [53]. These data showed that MALDI-TOF MS allows bacterial ID directly from infected urine in a short time, with great accuracy, and especially when Gram-negative bacteria with high bacterial counts are involved.

### 3. MALDI-TOF MS-based microbial typing and antibiotic resistance profiling

The robustness of the MALDI-TOF MS method is allowing microbiologists to obtain extremely stable proteomic profiles down to “strain” taxonomic level, despite high microorganism variability. Therefore, the range of applications of MALDI-TOF MS is constantly growing, from rapid species ID to labour-intensive proteomics on bacterial physiology (functional proteomics). So far, several studies have contributed to describe antibiotic resistance investigation performed by MALDI-TOF MS, with a thorough application into clinical microbiology pipelines, as reported in the pivotal paper by Hrabák et al. [87] (Fig. 5). The authors suggested that future perspectives should persist in three main directions, including detection of: i) antibiotic modifications by degrading enzymes; ii) resistance mechanism determinants through proteomics of MDR bacteria; and iii) modifications of target sites, such as ribosomal methylation. New approaches to study influx and efflux processes in bacterial cells should be based on quantification of antibiotics, opening new avenues for both experimental and clinical microbiology [87]. Recently, peptidic fingerprinting has been applied to highlight clonal distribution of MDR *Klebsiella pneumoniae* strains in both paediatric [77] and adult [88] nosocomial settings. In the paper by Bernaschi et al. [77], the proteomic-based typing was compared to other “typing” methods such as PFGE and automatic (repetitive-sequence-based-PCR) rep-PCR (DiversiLab System) in order to establish strain correlation indexes. Antibiotic susceptibility and phenotypic tests were also correlated by Berrazeg et al. [88] on 535 *K. pneumoniae* strains isolated in Algerian and French hospitals. The Main Spectra (MSP) dendrogram obtained from MALDI-TOF MS revealed five distinct clusters, significantly associated with respiratory infections/extended-spectrum beta-lactamase (ESBL) phenotype (Algeria), and UTIs/wild-type phenotype (France). In both papers MALDI-TOF MS was found to be a promising tool to identify and type *K. pneumoniae* strains according to their phenotypic properties and epidemiological distributions. Remarkably, MALDI-TOF MS can be nowadays complemented with functional proteomics assays to determine ESBL and carbapenemase activity in MDR strains of *E. coli* and *K. pneumoniae* [89] (Fig. 5). In some cases, as well as for anaerobic bacteria, susceptibility testing of clinical isolates is not considered recommended in routine clinical practice and the treatments are empirically established. However, periodic monitoring of susceptibility patterns of anaerobic bacteria is appropriate. Fingerprinting analyses, obtained by MALDI-TOF MS and automated rep-PCR, were reported in a recent paper [90] also on carbapenem resistance profiles of metallo-beta-lactamase (MBL) producing *Bacteroides fragilis* strains. A total of 830 clinical isolates of *B. fragilis* group were studied, with *B. fragilis* being the most prevalent species (59.5%). The susceptibility range of *B. fragilis* group isolates was represented by: penicillin; amoxicillin/clavulanic; piperacillin-tazobactam; cefoxitin; ertapenem; imipenem; clindamycin and metronidazole. The percentage of sensitive isolates did not change significantly over time for the entire set of molecules, with a slight increase in the resistance rate to ertapenem and imipenem. Six imipenem-resistant isolates were MBL-producing and PCR positive for cfa (imipenem-cefoxitin-hydrolyzing enzyme) gene. Both MALDI-TOF MS and automated rep-PCR, revealed a great genetic diversity amongst carbapenem-producing strains, suggesting the acquisition of novel resistance genes more than their clonal dissemination. Indeed, both methods appeared to be useful tools for fast and accurate ID and typing of *B. fragilis* group in the daily laboratory routine at strain level. Because of the relevant increase observed in *Bacteroides* species isolated also from blood cultures and the appearance of carbapenemase-producing strains [88], antimicrobial susceptibility, at least in the most severe patients, should be pursued in diagnostic pipelines. *Propionibacterium acnes* is an anaerobic skin commensal of relatively low pathogenicity. However, it is now emerging as an important opportunistic pathogen in many clinical situations, including late-stage prosthetic joint infections, osteomyelitis, endocarditis, endophthalmitis, and post-neurosurgical infections. At the population genetic level, *P. acnes* can be differentiated into a number of distinct phylogroups, known as types IA1, IA2, IB, IC, II and III, which may be associated with different types of infections and clinical conditions. The study by Nagy et al. [91] evaluated MS-based typing for resolution of these genetic groups after routine MALDI-TOF MS-based ID. A differentiating library was created to type 48 *P. acnes* clinical isolates and data were compared with multiplex sequence typing (MLST) results. Most of the clinical isolates (19/48) belonged to the type IA1 grouping, according to MALDI-TOF MS. By MLST, all isolates were identified as type IA1. Twenty-one clinical isolates belonged to the type IB cluster based on both MALDI-TOF MS and MLST typing. Eight clinical isolates were identified as type II strains by both typing methods and all the type III reference strains could be distinguished by the presence of a unique type III-specific peak (7238 Da) by the MALDI-TOF MS, hence indicating MS as a reliable and powerful tool for rapid ID/typing of *P. acnes* clinical groups [91]. Currently, early detection of outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) and adequate infection control measures are key objectives in hospital hygiene. In order to reach them, the potential of MALDI-TOF MS was compared to genetic typing methods such as PFGE, spa typing (S. aureus A protein) or MLST to describe epidemiologic relatedness of major clinical MRSA...
lineages [92]. By analysis of 25 MRSA isolate spectra, belonging to the 5 major hospital-acquired MRSA clonal complexes (CC5, CC8, CC22, CC30, CC45, as deduced from spa typing), reproducible spectrum differences were observed at 13 characteristic m/z values, allowing robust discrimination of the clonal complexes. Sixty independent clinical MRSA isolates, tested for presence or absence of the 13 characteristic MALDI-TOF MS peaks, provided 15 different profiles (MALDI types) and their hierarchical clustering showed high concordance with the clonal complexes [92]. Remarkably, these proteomic “types” may have the potential to become a valuable “first-line” tool for inexpensive and rapid typing of MRSA in infection control. In the study by Sun et al. [93], forty strains of S. aureus were investigated by both MALDI-TOF MS and API Staph ID system, testing performance of methicillin-resistance by Disk Diffusion Susceptibility Testing, to set a rapid method for MRSA assessment. Thirty-four strains were identified by MALDI-TOF MS as S. aureus, consistently with the API Staph ID system. Eleven strains were identified as MRSA and 23 as methicillin-susceptible S. aureus (MSSA). All 34 strains were therefore clustered into 2 groups by MALDI-TOF MS. The results were consistent with Disk Diffusion Susceptibility Testing, indicating MALDI-TOF MS as a rapid technique for MRSA ID and discrimination [93]. Also Burkholderia group is intrinsically resistant to many antibiotics and rapid ID is therefore crucial. However, reference MALDI-TOF MS databases are not suitable for reliable ID, especially for sibling species such as Burkholderia pseudomallei and Burkholderia mallei, and generation of dedicated libraries are therefore mandatory. In the paper by Karger et al. [44], a collection of ten B. pseudomallei and 17 B. mallei strains was used to generate a reference spectra library. Samples of both species were identified by MALDI-TOF MS, with a dedicated reference spectra library, but spectra demonstrated a higher homogeneity in B. mallei than in B. pseudomallei isolates. As expected for all closely related species, the ID/typing process with MALDI BioTyper software required a critical selection of spectra from reference strains [44]. When a dedicated reference set is used and spectra of high quality are acquired, very-closely related species can be unambiguously distinguished. Amongst bacteria causing foodborne illness, Yersinia enterocolitica is a Gram-negative coccobacillus-shaped bacterium, usually genotyped by exploiting the ail (attachment and invasion locus) gene target, used as target for the detection of pathogenic Y. enterocolitica strains in food testing. The all PCR does not detect, however, strains of biotype 1A (B1A), which are considered as non-pathogenic because lacking both virulence plasmid and chromosomally encoded virulence genes. However, in some recent reports, BT1A strains were described as harbouring the ail gene [63]. Whole-cell MALDI-TOF MS approach was applied to Y. enterocolitica and clearly classified the ail-positive biotype 1A strain within the cluster of BT1A strains, supporting PFGE-based strain patterns and genotyping analyses (ystB, ystA, virF and yadA virulence genes) of both pathogenic and non-pathogenic Y. enterocolitica strains and allowing a more comprehensive assessment of Yersinia strains pathogenicity [63]. Often, genetic and phenotypic heterogeneity require multiple-gene target combined typing methods (e.g., seven house keeping genes, atpD, gapA, gusA, mutM, nuoD, ppsA and recA). Amongst the others, the Stenotrophomonas genus is highly variable and, of the nine recognized species, only Stenotrophomonas maltophilia is of clinical importance relevance. Based on DNA-sequences, the genus encompasses “genogroups” of DNA-similarity below 97%, predominantly comprising strains of environmental origin. Therefore, to unveil the uneven distribution of environmental isolates within genogroups and reveal the genetic relationships within the genus, an easy and reliable approach is needed for Stenotrophomonas spp. ID. In the study by Vasileuskaya-Schulz et al. [94] a multi-locus sequence analysis (MLSA) was applied for the analysis of 21 S. maltophilia of environmental origin, Stenotrophomonas spp. and related genera. The genotypic outcomes were compared with MALDI-TOF MS typing. The MLSA provided reliable inter- and intra-species discrimination of all tested isolates that correlated with the MALDI-TOF MS data and provided the indication that some S. maltophilia genogroups of predominantly environmental origin are still to be classified [92]. MALDI-TOF MS typing seems to unravel also carbapenem resistance in Acinetobacter spp., appearing as a promising option to identify resistance patterns in routine use. In the paper by Alvarez-Buylla et al. [95], significant peaks for imipenem detection were identified, establishing optimal bacterial inoculums and test incubation time with and without dipicolinic acid and Zn2+, in order to distinguish between MBL and oxacillinases. Indeed, Acinetobacter genus contains 17 reference strains of Acinetobacter species and 7 genospecies (i.e., species 10 and 11), whose variability was analysed by MALDI-TOF MS, indicating that Acinetobacter bercziniae and Acinetobacter guillouiae represent two phenetically and phylogenetically distinct groups within the Acinetobacter genus. Despite their overall phenotypic similarity, the ability to oxidize D-glucose and grow at 38 ºC was employed in the differentiation of these two species. Interestingly, the strains of A. bercziniae originated mainly from human clinical specimens, whereas A. guillouiae strains were isolated from different environmental sources in addition to human specimens [96].

In the study by Böhme et al. [97], 70 reference strains from culture collections, including important seafood-borne pathogenic and spoilage bacterial species, and 50 strains isolated from commercial seafood, were analysed by both 16S rRNA sequencing and MALDI-TOF MS fingerprinting. The first analysis only identified the 50% of the strains at species level, appearing particularly poor at identifying Pseudomonas spp. and Bacillus spp. Conversely, MALDI-TOF MS identified 76% of the strains at species level. The MS spectral data were submitted to the SpectraBank database (http://www.spectrabank.org), performing peak mass cluster analysis by the web application SPECLUST and making the data available to the scientific community. Groupings appeared consistent with 16S rRNA-based phylogenetic results. Interestingly, for the MALDI-TOF MS analysis a higher discriminating potential was observed, especially for Pseudomonas and Bacillus genera, thus demonstrating to be an efficacious typing tool to extend phenotypic and genotypic approaches [97]. Nontypable H. influenzae (NTHi) and Haemophilus haemolyticus exhibit different pathogenicities; however, a reliable strategy for differentiating these strains is still lacking. In the study by Zhu et al. [30] MALDI-TOF MS was tested as a potential differentiating method combined to 16S rRNA and recombinase A (recA) gene analyses. The ORD was supported
Table 1 – Main studies on MALDI-TOF MS-based assays compared to reference molecular methods.

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<td>MALDI-TOF MS typing was concordant with reference methods</td>
<td>[77]</td>
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<tr>
<td>MALDI-TOF MS typing and antibiotic susceptibility tests</td>
<td>K. pneumoniae strains</td>
<td>535</td>
<td>Multi-locus sequence analysis (MLSA)</td>
<td>MALDI-TOF MS typing revealed five distinct clusters, associated with type of infections, country strain origin and antibiotic susceptibility</td>
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<td>Vitek-2, Phoenix automated systems and manual disk method for extended-spectrum β-lactamase (ESBL) phenotype; Hodge test and kpc gene PCR for carbapenemase susceptibility</td>
<td>Antibiotic susceptibility test MALDI-TOF MS-based determined ESBL and carbapenemase activity in concordance with reference methods</td>
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<td>MALDI-TOF MS typing</td>
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<td>Both MALDI-TOF MS and rep-PCR methods revealed a great genetic diversity amongst carbapenem-producing strains</td>
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<td>MALDI-TOF MS typing and antibiotic susceptibility tests</td>
<td>Propionibacterium acnes Methicillin-resistant Staphylococcus aureus (MRSA)</td>
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<tr>
<td>Rapid method for MRSA assessment based on MALDI-TOF MS typing</td>
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<td>40</td>
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<td>MALDI-TOF MS reference spectra library for Burkholderia spp.</td>
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<td>A careful selection of spectra from reference strains was required. When a dedicated reference set is used and spectra of high quality are acquired, it is possible to distinguish both species unambiguously</td>
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<td>MALDI-TOF MS typing for the assessment of Yersinia strains pathogenicity</td>
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<td>Acinetobacter spp.</td>
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<td>MALDI-TOF MS typing</td>
<td>Seafood-borne pathogenic and spoilage bacterial species</td>
<td>120</td>
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(continued on next page)
by a NRD, including specific Chinese strains for MALDI-TOF MS evaluation. The searching on ORD provided 76.9% of the NTHi (40/52) and none of the H. haemolyticus strains correctly identified at the species level. However, all NTHi and H. haemolyticus strains were accurately recognized at the species level by scanning NRD. Interestingly, geographically-dependent clustering was observed and, hence, Chinese and foreign H. influenzae strains were categorized into two distinct groups, with H. influenzae and H. haemolyticus also separated into two distinct categories. Compared to the existing methods, indeed, MALDI-TOF MS seems to have the advantage of integrating high throughput, accuracy, speed and consistency with epidemiological data [30] (Table 1).

4. MALDI-TOF MS-based ID and typing of yeasts

Recent studies on Candida spp. outbreaks have shown an increased incidence of bloodstream infections especially in neonatal intensive care units caused by Candida parapsilosis species, highlighting the need for their appropriate ID and epidemiological tracking. Several systems are available for molecular epidemiological and taxonomic studies of fungal infections: i) PFGE, still represents the gold standard for many microorganisms typing, including yeasts; ii) simple sequence repeats (SSRs) and iii) rep PCR, both based on repeated genome regions. Recently, MALDI-TOF MS has been widely used to identify and type microorganisms involved in nosocomial outbreaks. Indeed, robustness and reproducibility of MALDI-TOF MS profiles have been thoroughly described with reference to Candida species, body site, and antymycotic treatment [24]. In the study by Pulcrano et al. [98], 19 strains of C. parapsilosis, isolated from blood cultures of neonates, were genotyped by eight SSR markers and by MALDI-TOF MS. DNA- and MS-based results were compared in terms of discriminatory power to identify similarities amongst the isolates and study evolutionary changes in the C. parapsilosis population. The research showed that MS was a useful ID technique, also effective in monitoring the strain spread which is a key point for nosocomial infections control [98]. The Cryptococcus neoformans/Cryptococcus gattii species complex, a yeast-like fungus, comprises two sibling species, divided into eight major molecular types, C. neoformans VNI-VNIV and C. gattii VGI-VGIV. These genotypes differ in host range, epidemiology, virulence, antifungal susceptibility and geographic distribution. The current phenotypic and molecular IDs for species/molecular types often rely on real-time PCR-based methods, usually time consuming and expensive [99]. Recently, MALDI-TOF MS has been considered as an alternative for the rapid ID of C. neoformans and C. gattii at intra- and inter-species level. In a recent paper [100], 164 C. neoformans/C. gattii isolates, including four inter-species hybrids, were studied by MALDI-TOF MS and spectra correctly identified 100% of the isolates, grouped according to the currently recognized species, C. neoformans, C. gattii, and potential hybrids, and consistently with their major molecular types. Greater spectral differences were observed for C. neoformans than for C. gattii molecular types, most likely reflecting a closer phylogenetic relationship amongst the latter group. However, spectra were obtained not from intact yeast cells, but from FA-extracted cells, whose spectra were matched against an extended validated NRD, containing spectra of all eight major molecular types. Even in this case, MALDI-TOF MS can be considered a rapid ID tool for a correct recognition of the two currently recognized human pathogenic Cryptococcus species, characterized by high biological and taxonomic complexity [100] (Table 1).

5. Mono and polymicrobial communities: the case of blood cultures

It is nowadays clear that MALDI-TOF MS fingerprinting has become a powerful tool in microbiological diagnostics. MALDI-TOF MS also has the potential to directly identify pathogens in urine samples and blood cultures and, because of lengthier, more labour-intensive, and costlier techniques reserved for isolate ID in such samples, there is a drive to apply MALDI-TOF MS directly to samples from positive
blood-cultures and, in some cases, even to primary samples [23]. Indeed, this technology is expected to improve, as major challenge, especially the clinical management and guidance for chemotherapy. Sepsis is a life-threatening disease with high rates of morbidity and mortality worldwide; especially onco-haematological paediatric patients and newborns, particularly preterm infants, are at high risk of severe infections and sepsis due to their deficient and/or immature immunologic defences. Rapid ID of fungi or bacteria commonly causing sepsis could allow prompt species-specific therapy, leading to improved clinical outcomes and reduction of mortality, decrease of cost and length of hospitalization and lower development of antimicrobial resistance [101]. Blood culture is still considered the gold standard for the diagnosis of sepsis, allowing both ID and susceptibility testing. However, it suffers from several limitations, including lack of rapidity, requiring 1–3 days to become positive and additional 1–2 days for microorganisms ID and susceptibility testing. New diagnostic tests, that could rapidly and accurately identify bacterial and fungi species, are therefore required, including improvement of databases and softwares [102,103]. Several attempts have been made to identify bacteria from positive blood cultures, especially using molecular biology and real-time-based PCR. Rapid methods using microarrays, hybridization probes, and even flow cytometry techniques have been recently reviewed for diagnosis of bloodstream infections [104].

5.1. The pre-analytical step: the usage of homebrew methods

Several studies [105] have evaluated the contribution of MALDI-TOF MS for microorganism ID in positive blood cultures. The crucial pre-analytical step consists in separating the bacteria from cellular components in absence of which no ID is obtained. However, whatever is the protocol used, it seems that different centrifugation steps are needed, followed by lysis of blood elements to obtain reliable IDs. Nevertheless, different various drawbacks may be encountered such as correct ID of viridans Streptococci and Streplococcus pneumoniae and detection of the only predominant species in polymicrobial blood cultures [105]. These limitations highlight the importance of Gram staining to verify the presence of one or more microorganism morphology or stain, before performing MALDI-TOF MS. Ferroni et al. [106] reported a significant progress in sample preparation. Once the blood culture was identified as positive, a mild detergent was employed to lyse the cellular membranes within a few minutes, hence allowing bacterial ID in less than 30 min and canditating MALDI-TOF MS as the fastest of all techniques for bacterial ID directly from blood culture broths. In order to speed up the ID process, La Scola and Raoult [103], evaluated the bacterial ID directly from positive blood cultures. They prospectively analysed routine MALDI-TOF MS bacterial ID in blood cultures by two different protocols involving successive centrifugations and then lysis by TFA or FA. Out of the 562 blood culture broths detected as monomicrobic positive, 370 (66%) were correctly identified. Changing the protocol from TFA- to FA-based, Staphyloocci ID was improved, and complete correct ID increased from 59 to 76%. Lack of ID was observed mostly for viridans Streptococci. Amongst 22 positive polymicrobial blood culture broths, only one of the species was identified in 18 samples, no species were identified in two samples and false species IDs were obtained in two cases. Indeed, it seems that MALDI-TOF MS is an efficient method for direct routine IDs of bacterial isolates in blood cultures, with the exception of polymicrobial samples and viridans Streptococci [103].

5.2. The pre-analytical step: the usage of the Sepsityper™ kit

The performance of the MALDI Sepsityper™ kit for bacteria ID was evaluated by Kok et al. [107], applying manufacturer’s specified bacterial ID criteria (spectral scores ≥1.700–1.999 and ≥2.000 ID to genus and species level, respectively) and comparing results to standard phenotypic methods. Five hundred and seven positive blood cultures were examined, of which 379 (74.8%; 358 monomicrobial and 21 polymicrobial) were identified by MALDI-TOF MS; particularly, 132/195 Gram-positive (67.7%) and 149/163 Gram-negative (91.4%) from monomicrobial blood cultures were correctly identified at genus and species level, respectively. Spectral scores <1.700 (no ID) were obtained in 128/507 (25.2%) positive blood cultures, including 31.6% and 32.3% of Gram-positive and polymicrobial blood cultures, respectively. Significantly, more Gram-negative organisms were identified compared to Gram-positive organisms at species level (p < 0.0001). Five blood cultures were misidentified, but only at species level, including four monomicrobial blood cultures with Streptococcus oralis/mitis that were misidentified as S. pneumoniae. Therefore, a diagnostic algorithm for positive blood culture broths, including Gram staining and MALDI-TOF MS should identify the majority of pathogens, particularly at genus level [107]. In another study [108], Sepsityper™ kit was evaluated versus conventional separator gel columns: the MALDI-TOF MS-based ID of Gram-negative bacteria was significantly enhanced using the Sepsityper™ kit (99 versus 68% correct ID). For Gram-positive bacteria, 73% were correctly identified by MALDI-TOF MS with the Sepsityper™ kit and only 59% with the separator gel tube assay. A major problem of both methods was the poor ID of Gram-positive grape-like clustered cocci. As differentiation of S. aureus from coagulase-negative Staphylococci is of clinical importance, a PCR was additionally established, capable of identifying S. aureus directly from positive blood cultures, thus filling this diagnostic gap, with the additional value to detect MRSA in Staphylococci and vancomycin resistance in Enterococci [108]. Another study [109] compared Sepsityper™ kit to conventional method for bacterial preparation in order to perform susceptibility testing: no significant differences were observed in Gram-negative bacilli ID, regardless of the bacterial separation method used. However, ID was higher for Gram-positive cocci when the Sepsityper™ method was compared to an in-house method (ID 100% versus 84.15% for Staphylococci and ID 85.71% versus 57.14% for Enterococci, both at genus level). Therefore, the study suggested that Sepsityper™ method resulted more adequate for the further ID of Gram-positive cocci by MALDI-TOF MS [109]. Another protocol for bacterial ID from blood culture broths in hospital routine was assayed by Moussaoui et al. [110]. Before processing for bacterial protein extraction, 503 samples, examined over a three month period, were treated by Vacutainer gel separator tubes. Including polymicrobial samples, 193 of 213 of Gram-negative bacteria (91.08%) and 284 of 319 of Gram-positive
bacteria (89.02%) were correctly identified at species level. Enterobacteriaceae constituted 35.15% of all species found, Staphylococcaceae 37.96%, Streptococcaceae and Enterococcaceae 20.85%, Pseudomonadaceae 1.69% and anaerobes 2.44%. In most of the polymicrobial samples, only one species present was identified (80.9%). Seven isolates remained misidentified as S. pneumoniae, all belonging to S. mitis. S. aureus were better identified when grown on anaerobic-aerobic medium, and MALDI BioTyper ID scores as low as 1.4 were consistent in four successive outcomes for the same species. This protocol correlated with conventional microbiology procedures by up to 90%, and by >95% for only monomicrobial samples, definitely decreasing TAT for bacteria ID in blood cultures [110].

The study by Del Chierico et al. [111] evaluated the usage of MALDI-TOF MS on blood cultures (Microflex LT mass spectrometer, Bruker Daltonics), comparing MS-based IDs to conventional method Vitek 2 outcomes. With this aim, 200 blood cultures positive to BACTEC System (BD diagnostics) were Gram stained and cultured on solid media for microbe ID and antibiotic susceptibility by Vitek 2 (Biomerieux) or Phoenix (BD diagnostics). For MALDI-TOF MS ID, six ml from each bottle was centrifuged in gel Vacutainer tubes to separate microorganisms. The peptides were extracted by the standard ethanol/FA protocol and analysed by Microflex mass spectrometer (Bruker Daltonics). Generated spectra were processed by BioTyper software with scores associated with the correct ID probability. Results were compared to the reference biochemical systems and, when discordant, verified by MALDI-TOF MS on isolated colonies. Amongst the 200 analysed samples, 173 were monomicrobial cultures, of which 109 correctly identified by both methods, 19 discordant and 45 identified only by traditional techniques. Interestingly, the scores were reliable at species level for 57/109 samples, at genera level for 29/109, while a not reliable ID was obtained for 23/109. Fourteen blood cultures had polymicrobial growth and MALDI-TOF MS provided a correct ID for only one microbe in 11/14. Twenty-one out of the 200 isolates were identified as yeasts by Vitek-2, but only 3 were detected by MALDI-TOF MS. Particularly, 13 false positive samples on BACTEC system had no MALDI-TOF MS ID (Fig. 6). Therefore, also in this experimental setting, MALDI-TOF MS appeared a useful method to support routine ID of bacterial isolates from blood culture, because of its rapidity (around 45 min) and high sensitivity and specificity (65% and 72%, respectively).

![Fig. 6](image_url) – MALDI-TOF MS-based workflow for positive blood cultures designed by including both Sepsityper™ and homebrew protocols (e.g., gel Vacutainer). The conventional methods based on Gram staining and biochemical identification are compared with the two fast MALDI-TOF MS-based workflows. In the inset, the tables with ID results for each identified species are reported.
However, the method still shows a few limits: i) the score algorithm is not representative of the true ID; ii) failure of yeast ID; iii) in polymicrobial samples microscope discrimination is not achieved; iv) the sensitivity is affected by the spot loading capacity (Fig. 6) [111]. Additionally, a comparison between gel Vacutainer and MALDI Sepsityper™-based extraction methods performed on further 50 positive blood cultures, revealed 38/50 and 45/50 correct IDs, respectively, with a sensitivity increment of 18.4% obtained by using the MALDI Sepsityper™ kit (Fig. 6) [111]. New methodological approaches also developed sample enrichment into specific growing media before extraction process and prior to MS analysis [112].

In the recent study by Chen et al. [113], the performance of two commercial MALDI-TOF MS systems, VITEK MS IVD (bioMerieux) and Microflex LT BioTyper (Bruker Daltonics), was evaluated on direct bacterial ID on positive blood cultures with the use of the commercial MALDI Sepsityper™ extraction method. Amongst the 181 monomicrobial cultures analysed, both systems generated genus to species level IDs amongst >90% of specimens (BioTyper: 177/181, 97.8%; VITEK MS IVD: 167/181, 92.3%). Generally, the BioTyper system generated significantly more accurate IDs than the VITEK MS IVD system for the monomicrobial cultures and was able to identify the minority species amongst polymicrobial blood cultures. An in-house extraction method was also evaluated versus the Sepsityper™ kit on both MALDI-TOF MS systems. The in-house method could generate more correct IDs at genus level than the Sepsityper™ kit (96.7% versus 93.5%) on the BioTyper system, whereas they exhibited the same performance (88.0%) on the VITEK MS IVD system. The study by Chen et al. [113] confirmed the practical advantages of MALDI-TOF MS and their “in-house” extraction method, which reduced the reagent cost to US $1/sample and the TAT to 3 h, highly effective indexes for a diagnostic microbiology service [112].

5.3. The post-analytical step: the ID processing algorithm

Post-analytical modifications of ID cut-off scores may also improve diagnostic accuracy. In the paper by Nonnemann et al. [114], a cut-off score of 1.5 was considered and compared to the recommended 1.8. The 3-month study on positive blood cultures was carried out to evaluate whether the Sepsityper™ Kit with BioTyper 2.0 software could be used as a fast diagnostic tool for bacteria and fungi and whether a 1.5 cut-off score could improve species IDs, compared with the recommended score of 1.8. Two hundred and fifty-six positive blood cultures from 210 patients and 19 blood cultures spiked with fungi were examined. Using the cut-off score of 1.8, 81% Gram-negative bacteria were identified at species level compared to 84% using a cut-off score of 1.5. For Gram-positive bacteria, 44% were identified at species level with a cut-off of 1.8, compared to 55% with the 1.5 value. The entire ID rate was 63% (cut-off 1.5) and 54% (cut-off 1.8); 77% of fungal species were identified with both log scores. The recent study confirmed the MALDI-TOF MS as a reliable fast diagnosis tool for Gram-negative bacteria and fungi but lesser for Gram-positives. However, the 1.5 cut-off score increased the diagnosis for both Gram-positive and -negative bacteria [114]. Lastly, results obtained so far by MALDI-TOF MS applied to direct bacterial ID on positive blood cultures have been encouraging but have also shown some limitations, mainly related to bacterial growth and the presence of interfering substances belonging to blood cultures. Even if it is highly efficient now, after a decade of experiments, the technique is currently still being improved. However, it lacks standardization and exhaustive databases and more efficient softwares are necessary. Thus, it is likely that bacterial IDs from complex matrices, such as blood cultures and urines, will become more and more accurate in the near future.

6. Proteomics and metaproteomics of microbial ecosystems

The proteomic analysis of complex microbial communities is a new promising research field aiming at assessing the “bioreactor” features of a microbial community. Description of gut microbiota phylotypes needs employment of original “metaproteomic” pipelines, implemented by appropriate non-redundant repository databases for interrogation and operational taxon units (OTUs) inference. However, axenic culture-based MALDI-TOF MS IDs may still act as reference and corroborating outcome for metaproteomics-obtained OTUs (Del Chierico et al., 2013, JPROT-D-13-00094, Accepted). Indeed, the term “metaproteomics” was coined only in 2004 by Wilmes and Bond [115] for large-scale ID of the whole protein content of an environmental community of prokaryotic microorganisms. In this pivotal study, successful extraction and purification of the entire proteome enabled its large-scale characterization by 2D-PAGE, quadrupole TOF MS and de novo peptide sequencing analysis. Afterwards, Ram et al. [116] conducted a very comprehensive metaproteomic study combining “shotgun” MS-based proteomics analysis with gene expression techniques to evaluate in situ microbial activity of a low complexity natural microbial biofilm. In this experimental work, they characterized more than 2000 proteins, of which 215 were novel, from the most abundant microorganisms. A rich literature has provided a lot of information into low complexity microbial communities [117-119], human saliva [120], human urine [121], marine [122], environmental [123] and human faecal samples [124-128].

Although it is still an early approach, metaproteomics may have the potential to provide extensive new functional information for diverse ecosystems and especially for the principal microbe–host ecosystem which is the human gut. In this regard, metaproteomics has functionally characterized the microbiota in the developing human infant GI tract [124], and discovered temporal stability of a core proteome for an established intestinal microbiome of an adult human [128]. Verberkmoes et al. [125], realized a high level of metaproteome characterization, focusing on the unaltered adult human gut microbiomes for two healthy subjects, highlighting the strong integrated relationship between microbial and human proteins [125]. Finally, Erickson et al. [126], integrating metaproteomics and metagenomics, characterized the human host-microbiota signatures of human
Crohn’s disease [126]. However, challenges for metaproteomic investigations include irregular species distribution, dynamic range of protein expression levels within microorganisms, and large genetic variety within the microbial communities [129]. In spite of these difficulties, metaproteomics has the great potential to link the genetic multiplicity and activities of microbial communities with their impact on the ecosystem function, but needs to deal with the cornerstone culturomics [130,131] and the metagenomics based on “next generation sequencing” (NGS) evidence.

7. Outlook on other MALDI-TOF MS applications and future developments

Traditional microbial typing technologies for the characterization of pathogenic microorganisms and monitoring of their global spread are often difficult to standardize and poorly portable; they also lack sufficient ease of use, throughput and automation. To overcome these problems, the use of comparative sequencing by MALDI-TOF MS for automated high-throughput microbial DNA sequence analysis has been included into pathogen ID and characterization pipelines and has been implemented. For example, data derived from the public MLST database (http://pubmlst.org/neisseria) were utilized in the study by Honish et al. [132] to establish a reference set of expected peak patterns. A model pathogen, Neisseria meningitidis, was used to validate the technology and explore its applicability as an alternative to dideoxy sequencing (DS). One hundred N. meningitidis samples were typed by comparing MALDI-TOF MS fingerprints of the standard MLST loci to reference sequences available in the public MLST database. IDs were obtained in two working days. Results were in concordance with classical DS with 98% correct automatic ID. Sequence types (STs) of 89 samples were represented in the database, seven samples revealed new STs, including three new alleles, and four samples contained mixed populations of multiple STs. The approach showed interlaboratory reproducibility and allowed for the exchange of MS fingerprints to study the geographic spread of epidemic N. meningitidis strains. The Sequenom MassARRAY iPLEX single-nucleotide polymorphism (SNP) typing platform MALDI-TOF MS, coupled with single-base extension PCR for high-throughput multiplex SNP detection, was investigated for S. aureus MRSA genotyping, by developing a 16-plex MassARRAY iPLEX GOLD assay (MRSA-iPLEX) [133]. The method was evaluated with 147 MRSA isolates, and the results were compared with those of an established SYBR Green-based real-time PCR system utilizing the same SNP-binary markers. A total of 2352 markers belonging to 44 SNP-binary profiles were analysed by both real-time PCR and MRSA-iPLEX. With real-time PCR as the reference standard, MRSA-iPLEX correctly assigned 2298 of the 2352 (97.7%) markers. MRSA-iPLEX provided optimal throughput for MRSA genotyping and was, on a reagent basis, more cost-effective than the real-time PCR methods. Therefore, the 16-plex MRSA-iPLEX represented a suitable alternative to SYBR Green-based real-time PCR typing of major sequence types and clonal complexes of MRSA. In the study by Bouakaze et al. [134], the potential use of another SNP genotyping Sequenom was investigated for the simultaneous analysis of 16 SNPs, previously validated as a useful tool for Mycobacterium tuberculosis complex (MTBC) species ID and for the classification of MTBC isolates into distinct genetic lineages (principal genetic groups and SNP cluster groups). In this context, the authors developed a 16-plex iPLEX assay based on an allele-specific-primer single-base-extension reaction using the iPLEX Gold kit (Sequenom), followed by MALDI-TOF MS analysis on the commercially Sequenom MassARRAY platform. This assay was tested on a panel of 55 well-characterized MTBC strains that were also genotyped for the same loci using the previously reported SNaPshot assay, as well as 10 non-MTBC Mycobacteria and 4 bacteria not belonging to the Mycobacterium genus. All MTBC samples were successfully analysed with the iPLEX assay, which yielded clear allelic data for 99.9% of the SNPs (879 out of 880). No false-positive results were obtained with the negative controls. Compared to the SNaPshot assay, the newly developed 16-plex iPLEX assay produced fully concordant results that allowed reliable differentiation of MTBC species and recognition of lineages, thus demonstrating its potential value in diagnostic, epidemiological, and evolutionary applications. Compared to the SNaPshot approach, the implementation of the iPLEX technology could offer a higher throughput and could be a more flexible and cost-effective option for microbiology laboratories.

In another study [135], a new multiplexed primer extension-based spoligotyping assay using MALDI-TOF MS improved the classical reverse line blot hybridization assay (RLHA) with respect to reproducibility, throughput, process flow, ease of use, and data analysis. Validation of the MALDI-TOF MS-based spoligotyping assay with two sample sets (i.e., totally 326 samples), resulted in 96.6% concordance (315/326), when the full spoligotype patterns were compared with the results of standard spoligotyping, and 99.9% concordance, when the results were compared with those of individual primer extension assays. Ten strains showed discordant results with one or two spacer differences from the membrane-based spoligotyping results. Most discordant samples were identified to be the outcome of ambiguities in the interpretation of weak hybridization signals in the RLHA and sequence variations in the spacer regions. The authors established a new automated primer extension assay and successfully validated it for the use on the routine typing of MTBC strains in the research and public health laboratory environments, possibly extendable to antibiotic resistance markers [135].

During the past five years, MALDI-TOF MS has become a powerful tool for routine IDs in many clinical laboratories. Seng et al. [136] analysed 11-year experience in routine ID of clinical isolates (40 months using MALDI-TOF MS and 91 months using conventional phenotypic identification, CPI). Amongst the 286,842 clonal isolates, 284,899 isolates of 459 species were well-identified. The remaining 1951 isolates were misidentified and required confirmation using a second phenotypic ID for 670 isolates and a molecular technique for 1273 isolates of 339 species. MALDI-TOF MS annually identified 112 species, i.e., 36 species/10,000 isolates, compared to 44 species, i.e., 19 species/10,000 isolates, for CPI. Only 50 isolates required second phenotypic IDs during the MALDI-TOF MS period (i.e., 4.5 re-IDs/10,000 isolates) compared with 620 isolates during the
CPI period (i.e., 35.2/10,000 isolates). Remarkably, 128 bacterial species rarely described as human pathogens were reported, including 48 phenotypic techniques (22 using CPI and 37 MALDI-TOF MS). Another 75 rare species were identified using molecular methods. Therefore, MALDI-TOF MS reduced the time required for ID by 55-fold and 169-fold, and the cost by 5-fold and 96-fold, compared with CPI and gene sequencing, respectively.

Recently, whole-cell MALDI-TOF MS has also allowed the ID of mammalian cells such as macrophages. The whole-cell MALDI-TOF spectra of macrophages stimulated with IFN-γ and IL-4, produced spectra with peaks ranging from 2 to 12 kDa, very different from those of unstimulated and stimulated macrophages. Interestingly, the fingerprints induced by M1 agonists (i.e., IFN-γ, TNF, LPS and LPS + IFN-γ) and M2 agonists (i.e., IL-4, TGF-β1 and IL-10) were specific and readily identifiable. Thus, whole-cell MALDI-TOF MS was able to characterise M1 and M2 macrophage subtypes, but also fingerprints induced by extracellular or intracellular bacteria were bacterium-specific. This approach is opening a new avenue to study immune response in the clinical setting, by monitoring the various activation patterns of immune cells in pathological conditions [137].

The ability to rapidly identify bacterial species, rarely described as pathogens in specific clinical specimens, will help us to study the clinical burden resulting from the emergence of these species as human pathogens, definitely suggesting MALDI-TOF MS an alternative to molecular methods in clinical laboratories [136]. Further implementations in sample processing, enrichment and interpretative ID score algorithms still need to be accomplished, making MALDI-TOF MS the future shared automated platform in clinical microbiology and the robust and reference reservoir for NGS data generation and interpretation.

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