microbiology research

MDPI

Review

The Current Level of MALDI-TOF MS Applications in the Detection of Microorganisms: A Short Review of Benefits and Limitations

Ali Haider [1], Marianna Ringer [2], Zsolt Kotroczó [3], Csilla Mohácsi-Farkas [4], and Tamás Kocsis [4,*]

[1] Doctoral School of Food Sciences, Hungarian University of Agriculture and Life Sciences, H-1118 Budapest, Hungary
[2] Geographical Institute, Research Centre for Astronomy and Earth Sciences, H-1112 Budapest, Hungary
[3] Department of Agro-Environmental Studies, Hungarian University of Agriculture and Life Sciences, H-1118 Budapest, Hungary
[4] Department of Food Microbiology, Hygiene and Safety, Hungarian University of Agriculture and Life Sciences, H-1118 Budapest, Hungary
* Correspondence: kocsis.tamas.jozsef@uni-mate.hu

Abstract: The available literary data suggest the general applicability and benefits of the Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) in the field of microbiological identification. Due to its high reliability, MALDI-TOF might generally be the alternative to the the16s-rRNA sequence-based and serological-based methods. The essence of the technique is to map the unique protein pattern of microbes that contributes to characterizing a wide variety of microorganisms, including bacteria, fungi, and viruses. With its application, the well-known bacterial and fungal species can be quickly identified, thus saving time in clinical diagnostics. In recent years, new protocols have appeared for directly identifying pathogenic strains from patient samples (blood, urine, feces), a major milestone in healthcare applications. On the other hand, these applications only have reliable results under certain conditions (homogeneous infection, adequate cell count, appropriate separation technique). This review aims to introduce and summarize those developments that have been enabled for routine application in the field of clinical diagnosis.

Keywords: identification; quantitative MALDI-TOF; pathogens; diagnostics

Citation: Haider, A.; Ringer, M.; Kotroczó, Z.; Mohácsi-Farkas, C.; Kocsis, T. The Current Level of MALDI-TOF MS Applications in the Detection of Microorganisms: A Short Review of Benefits and Limitations. *Microbiol. Res.* **2023**, *14*, 80–90. https://doi.org/10.3390/microbiolres14010008

Academic Editors: Beniamino T. Cenci-Goga

Received: 30 November 2022
Revised: 13 January 2023
Accepted: 18 January 2023
Published: 19 January 2023

1. Introduction

Identifying microorganisms (such as bacteria, yeasts, and molds) by physiological, serological, biochemical, and chemotaxonomic methods usually requires much effort, resources, and time. The diagnostic includes assessing the shape of the colony and applying phenotypic and biochemical testing, especially in the case of fungi, which is often determined based on their distinctive microscopic and macroscopic shapes [1]. Although the reliability of more recently applied genomic methods, such as 16S rRNA gene sequence analysis and multilocus sequence analysis, is high, they cannot provide quick results. In addition to being time-consuming, these techniques require extensive experience and training to achieve accurate identification. However, new technologies for the accurate and rapid identification of bacteria are essential in various fields of applied microbiology. Mass spectrometry is an alternative solution for identifying and strain typing. This analytic technique can analyze the mass-to-charge ratio of numerous biomolecules, such as peptides and proteins [2]. The method currently used for this purpose is matrix-assisted laser desorption ionization (MALDI). The essence of the MALDI measurement is that the molecules of the examined sample are ionized with the help of an auxiliary material (ma-

trix) that can absorb the excess laser energy used for ionization [3]. The analytes are embedded in the crystal of the matrix, which transfers the laser energy to the macromolecules of the analyte [4]. During the process, the macromolecule-matrix complexes from the test sample are released (desorption phase), and then the resulting molecular ions are delivered to the analyzer under high vacuum and accelerating voltage (Figure 1).

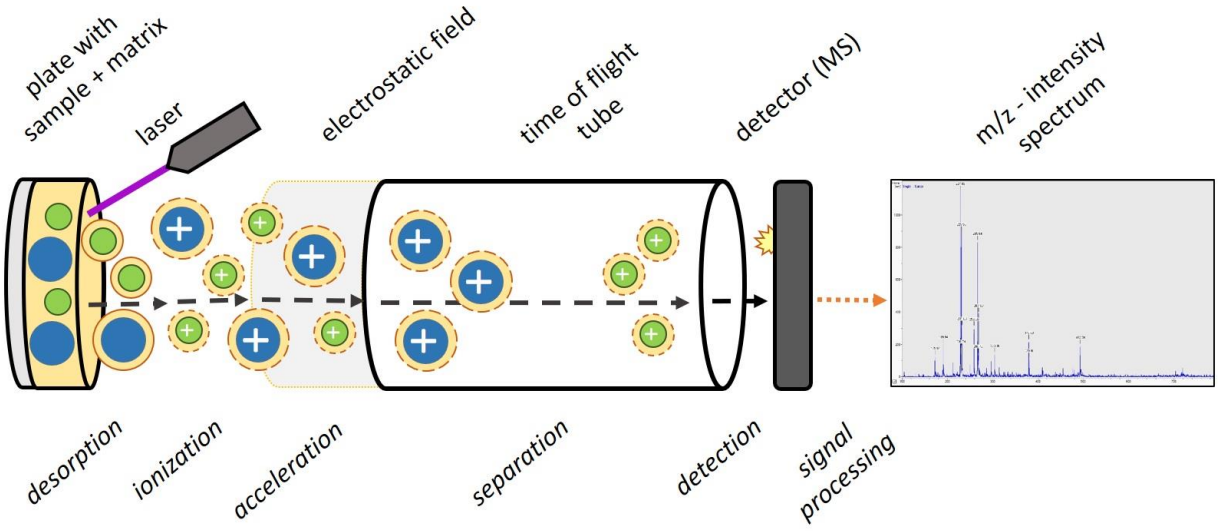

Figure 1. Overview of the investigation process. The sample–matrix complex is evaporated and ionized by laser irradiation. The ions are accelerated in an electric field and drift in a field-free pathway under a vacuum. During the flying, a separation between low-mass and high-mass ions occurs. The flight time depends on the length of the flight path, the ion's mass, its energy, and the value of charges.

The first description of using MALDI mass spectrometry technology for bacterial biomarkers was published in 1975 [5]. Still, it took a long time to introduce this technology in routine microbiology. Over and above in 2004, the first complete database for bacterial identification was reported [6]. At the same time, it was quickly recognized that this technique is also suitable for examining the unique protein pattern of microbes [7]. The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is now a widely used technique to characterize a wide variety of microorganisms, including bacteria, fungi, and viruses [8]. A multicenter evaluation study of the MALDI-TOF MS system for identifying gram-negative bacteria was performed, including a total of 2.263 isolates representing 23 genera and 61 species. The study showed that the MALDI-TOF MS system correctly identified 99.8% at the genus and 98.2% at the species level [9]. Spanu et al. evaluated the use of MALDI-TOF MS for identifying the most relevant species of *Staphylococcus*, using the *rpoB* gene sequencing method as a reference. Correct species identification was achieved in 99% of strains until the subspecies level [10]. Handal et al. aimed to evaluate the reliability of identification by MALDI-TOF MS compared to 16S rRNA sequencing of the most common clinically relevant anaerobic bacteria, including *Bacteroides* spp., *Clostridium* spp., *Prevotella* spp., *Fusobacterium* spp., and gram-positive anaerobic cocci. Authors reported that the MALDI-TOF MS correctly identified about 95% of the anaerobes to the genus level, and 87% to the species level, with identification errors mainly among the non-*fragile Bacteroides* spp. and the gram-positive anaerobic cocci. MALDI-TOF proved to be a successful method for identifying anaerobes [11]. MALDI-TOF MS, which can measure peptides and other compounds to analyze their complex mixture, is an ideal method for measuring non-purified extracts and intact bacterial cells (Biotyper). It is a rapid, accurate, and cost-effective way of microbial characterization and identification compared to classical and molecular ways. During the measurement, mass

spectral fingerprints generate from the sample's protein content, unique signatures for each microorganism at the species level (Figure 2).

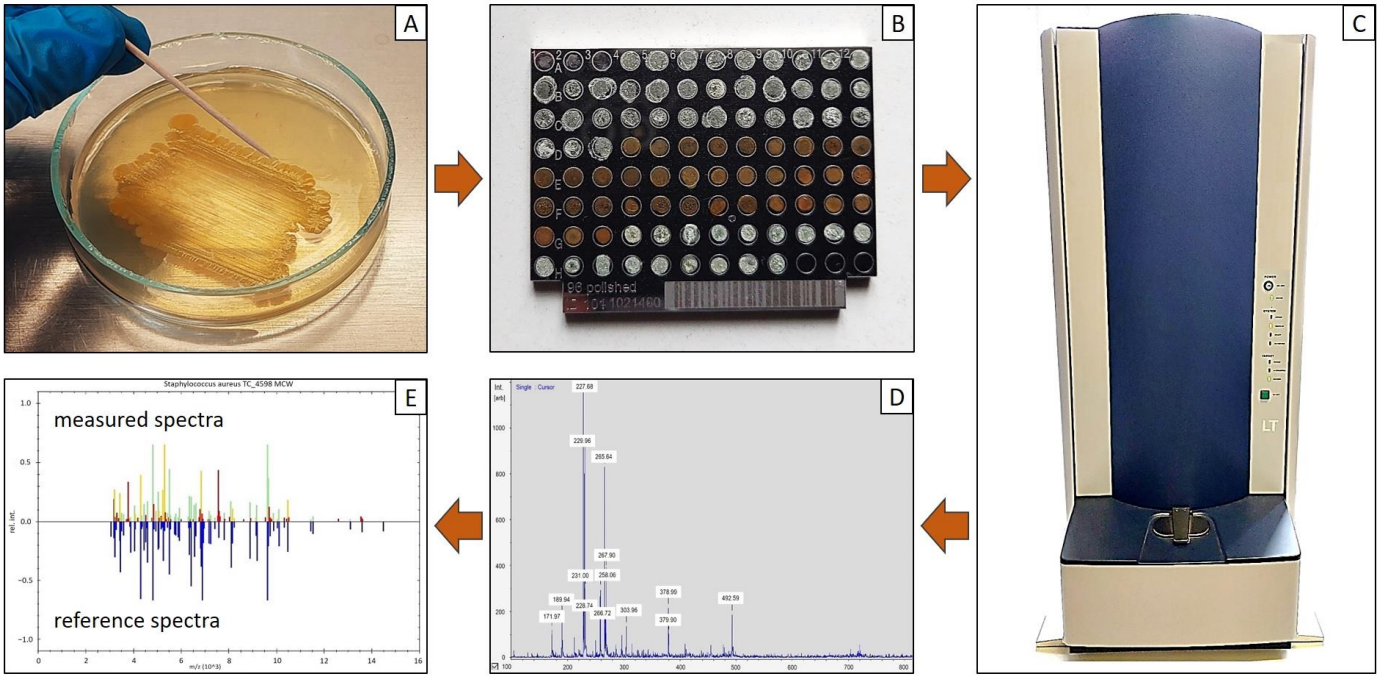

Figure 2. Workflow of sample preparation and profile analysis by MALDI-TOF Biotyper. (**A**) Sampling from the colony, (**B**) Preparation on MALDI Target plate, (**C**) Instrumental measurement, (**D**) Generating mass spectra, (**E**) Calculating MALDI-TOF profile spectra. During the measurement, the generally known peaks are identified, and their pattern is compared with the reference list in a database.

Since the MALDI-TOF MS is a suitable, rapid, and low costs technique with the capability of analyzing a high sample volume simultaneously, it can be the alternative to conventional biochemical and molecular identification systems in laboratories. Although the reliability of the identification results of microbial cultures is remarkable, it is still unclear how efficient the MALDI is in the case of diagnostic purposes. Is it possible to directly identify a large number of microbes in the samples, for example, milk, blood, urine, or feces? Another question: does the spectral analysis have the potential for strain typing in investigating the spread of pathogens?

2. Approach to the Methodological Concept

In recent years, MALDI-TOF MS can be found in routine laboratories and utilized as an alternative approach for identification. During the preparation process, the sample can practically be picked up with a sterile toothpick and prepared on a target plate, which should be covered with 1 µL formic acid. When dried, it is overlayed with 1 µL α-HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) matrix solution and left to dry again [12]. After the crystallization of the matrix-analyte mixture on the target plate, it is bombarded with short laser pulses, usually from a UV/Vis laser. The matrix absorbs the laser energy, leading to the desorption of the analytes, which are then vaporized and ionized into the gas phase. This matrix-assisted desorption and ionization of analytes lead to the formation of predominantly singly charged sample ions. The desorbed and ionized molecules are first accelerated by an electrostatic field and then ejected through a flight tube subjected to a vacuum until they reach the detector. The time of flight (TOF) required to reach the detector depends on the mass (m) and charge (z) of the analyte and is proportional to the square root of m/z [13]. Thus, bioanalytics with different m/z that make up a complex sample is separated according to their TOF, creating a

mass spectrum characterized by both m/z and ion intensity, corresponding to the number of ions. Based on this mass spectra information, a characteristic fingerprint can be recorded of organic matter that can be investigated typically between 2000 and 20,000 m/z. In this range, the signal-to-noise ratio is very stable and easily detectable [14]. Generally, MALDI produces singly charged (z = 1) ions, so the m/z value of the analyte corresponds to its mass plus cation adduct. [15].

3. Identification reliability of MALDI-TOF

For the past few years, MALDI–TOF MS has been used to identify various microorganisms, for example, Gram-negative rods (e.g., *Escherichia coli* and other members of the *Enterobacteriaceae* family) [16], Gram-positive cocci (e.g., *Staphylococcus aureus* and *Streptococcus*) [17], and, some Gram-positive rods (e.g., *Bacillus cereus*) [18]. Many extensive studies assessing the ability of MALDI–TOF MS to identify bacterial strains isolated from clinical samples have been published. The first one showed that, at the species level, MALDI–TOF MS accurately identified 84.1% of the 1,660 strains tested [19]. A retrospective study by Eigner et al. [20] on 1116 routine isolates representing the main bacterial groups encountered in the clinical microbiology laboratory showed 95.2% correct identification by MALDI-TOF MS.

Cherkaoui et al. (2010) [21] evaluated the two main MALDI-TOF MS systems, Bruker and Shimatzu, in a comparative study with 720 bacterial isolates under routine clinical laboratory conditions. The isolates were analyzed in parallel on both devices according to the manufacturer's default recommendations. The MALDI-TOF MS results were compared with conventional biochemical identification tests, and discordant results were resolved with 16S rRNA gene sequencing. The Bruker MS system gave high-confidence identification for 680 of 720 isolates (94.4%), whereas the Shimadzu MS showed a high-confidence identification for 639 isolates (88.8%). These results also showed that only 6/680 (0.9%) of the Bruker and 3/639 (0.5%) of the Shimadzu identifications gave an incorrect high-confidence identification at the species level. All the high-confidence MS identifications were accurate at the genus level. In addition, Bruker's Biotyper software package has identified 9 (69%) and the Shimadzu Corporation Axima Assurance system coupled with the SARAMIS database 5 (38%) of 13 isolates that were not identified by conventional phenotyping methods.

4. Detection of Infection Directly from Blood Culture by MALDI-TOF

Bloodstream infection, septic shock, and endocarditis represent severe diseases with important mortality and morbidity. Blood culture represents the best way to establish the etiology of such infections and to guide antimicrobial treatment. This is important since rapid and appropriate antimicrobial therapy is pivotal to reducing poor outcomes [22]. Indeed, the fatality rate was 20% for bloodstream infection patients treated with appropriate therapy and 34% for patients treated with inappropriate therapy [23].

In situations where it is unclear which particular organs are affected, blood cultures are useful for obtaining information concerning the infecting organism. The use of MALDI-TOF MS for identifying bacteria requires a bacterial abundance above a certain threshold; therefore, this method is often used after confirming colony formation. However, in cases where speed is particularly essential, it is possible to perform MS-based ID directly using a culture solution as soon as the blood culture is considered positive. This is one of the most promising technologies currently available for identifying microbial pathogens directly from positive blood culture bottles [24,25] and has considerable significance as a rapid diagnostic method, reaching a positive predictive value of 60–80% for different species of microorganisms [26]. Differentiating microorganisms from host cells is a critical step for successful ID, and several laboratory-developed and commercially available protocols have been reported for this purpose, as reviewed elsewhere [27,28]. The following are representative examples of laboratory-developed test protocols: (1)

stepwise sedimentation of blood cells and microorganisms [29]; (2) low-speed centrifugation for removing blood cells, followed by an additional lysis procedure [28]; (3) removal of blood cells using serum separator tubes [30]; (4) saponin use [31]. At the same time, there are three commercial protocols currently available: the Sepsityper® kit (Bruker Daltonics, Bremen, Germany) [32], the VITEK® MS blood culture kit (bioMérieux, Budapest, Hungary) [33], and the rapid BACpro® II kit (Nittobo Medical Co., Tokyo, Japan) [34,35]. In clinical samples, multiple proteins may be present in high quantities that do not come from bacteria, such as hemoglobin in blood cultures; therefore, pretreatment steps, such as the separation of blood cells, are required to recover the bacterial cells selectively [28]. Quick bacterial ID by MALDI-TOF MS on blood culture material enables rapid administration of relevant treatment to the patient, resulting in decreased time spent in intensive care units and length of hospitalization [36–38].

Maier et al. (2008) analyzed 54 clinical samples directly from blood cultures, which resulted in 41 identifications (75.9%), while two additional samples were correctly identified but with a reliability score below the given threshold for species identification. Three sample identifications were discordant on the species- but identical on the genus level, while eight blood cultures (14.8%) did not allow a successful direct identification [39].

5. Detection of Infection Directly from Urine by MALDI-TOF

Recent studies aimed at applying MALDI-TOF MS technology directly on urine samples suggested promising results if the urine contains more than 100,000 CFU/mL [40]. To improve the sensitivity of MALDI-TOF MS when performed directly on clinical samples, specific protocols based on membrane filtration and magnetic separation to collect the bacteria and obtain an enriched solution for MS were employed that have improved the detection sensitivity of MALDI-TOF MS to 1000 CFU/mL [41]. Notably, urinary tract infections (UTIs) are humans' most frequent type of bacterial infection. In a study of 220 urine samples in which monomicrobial bacterial growth was higher than 105 CFU/mL, the organism could be identified to the species level in 202 of the samples (91.8%) [42]. Veron et al. compared three ID methods (differential centrifugation, urine filtration, and 5 h bacterial cultivation on solid culture media) based on their ability to identify bacteria and their potential as a routine tool for microbiology laboratories [43]. A higher proportion of correct MALDI-TOF MS bacterial ID was obtained through filtration (78.9%) and the culture-based method (84.2%) compared with centrifugation (68.4%) [40].

In another experiment, Schwarz et al. (2008) analyzed 37 urine samples [44]. In 33 cases, the analysis resulted in identifications that were confirmed by serological tests as well. Four samples found to contain two microorganisms after culturing were not identified by the direct MALDI approach. The complete direct identification process took about one and a half hours from preparation to analysis. Thereby, the time required for identification was shortened by at least 24h compared to traditional culture-dependent procedures. These results demonstrated that a short culture step is a straightforward and efficient sample preparation method, enabling the fast and reliable ID of uropathogens by MALDI-TOF MS.

6. Detection of Milk Microbial Contamination by MALDI-TOF

MALDI-TOF MS has been applied in the field of veterinary medicine as well. MALDI-TOF MS is becoming more commonplace for the genus- and/or species-level identification of bacteria isolated from milk samples and, in some laboratories, has replaced conventional biochemical methods.

Wilson et al. (2019) [45] cultured milk samples submitted from a commercial dairy farm from recently calved cows or clinical mastitis cases. They identified 181 isolates using conventional biochemical testing, MALDI-TOF MS, and 16S ribosomal DNA sequencing analysis. The positive agreement among all three diagnostic methods was 94%, with 95% to 98% between each pair of methods. The overall (including negative agreement) agreement among all 3 methods ranged from 97% to 100%. The results of the present study

suggest that when identifying pathogens at the genus or species level, conventional culture followed up with either secondary biochemical testing or MALDI-TOF MS is of practical value. For milk quality and udder health monitoring or research, any of the 3 methods is a valuable tool for genus-level identification of bacteria isolated from dairy cow milk [45]. In another experiment, Pukančíková et al. (2016) reported that the dominant microbial genus of raw cow milk was *Pseudomonas*. Besides that, microbial genera *Aeromonas*, *Candida*, *Corynebacterium*, *Enterococcus*, *Hafnia*, *Kocuria*, *Kytococcus*, *Lactococcus*, *Micrococcus*, *Raoultella*, *Acinetobacter*, *Citrobacter*, *and Sphingobacterium* were presented and identified in raw milk as well. The main producers of spoilage enzymes were *Candida inconspicua*, *Kocuria rhizophila*, *Raoultella ornithinolytica*, *Acinetobacter johnsonii*, and *Citrobacter braakii* [46]. They isolated 30 (6 psychrophilic, 11 mesophilic, and 4 thermophilic) pure cultures from raw cow milk on MRS and MPA agar plates identified by MALDI-TOF MS. The 9 isolates, differing in micro and macroscopic properties after cultivation on MRS and MPA plates, were identified as the same species. Based on that, the microbiota of raw milk can typically contain several species, which draws attention to the risk of false identification results.

7. Utilization of MALDI-TOF in the Detection of Anaerobic Bacteria

Anaerobic bacteria exist as part of the normal flora in the human intestinal tract, oral cavity, and urogenital tract [47]. They can cause infectious diseases due to impairment of the microenvironment and/or immune system. Anaerobic infection can also be induced by deep wounds accompanied by facultative anaerobes and aerobic bacteria invasion. Invasive anaerobic infections are life-threatening, and the mortality rate of anaerobic bacteremia is high as 40% [48]. Thus, the accurate and fast identification of anaerobic bacteria is pivotal to prompt antimicrobial treatments. Conventional anaerobe identification methods are cumbersome, time-consuming, and costly. It requires long-term cultivation (not less than 24 h) to obtain enough inocula. In addition, the identification work is complex, including colony traits, colony morphology, and staining results. Meanwhile, it is difficult to identify rare or newly identified species using conventional phenotyping methods and commercial kits [49]. Real-time, fast, high-throughput, high-sensitivity, high-selectivity, and low cost have been the goals analysts pursue in modern analytical science.

A study was conducted by Lau et al. (2014) with 28 anaerobic genera included and assessed critically using two currently available MALDI-TOF MS systems [49]. It is known that anaerobes are more difficult to be identified in clinical laboratories [50]. However, using MALDI-TOF MS, the overall identification accuracy of anaerobic bacteria was 92% at the genus level in 28 included articles with 6685 various anaerobes isolates. These results indicate that MALDI-TOF MS is a qualified method for the accurate and rapid identification of pathogenic anaerobes. At the same time, it was noticed that the identification property of MALDI-TOF MS against common anaerobe isolate species was variable. Among them, the correct rate was more than 80% for 18 anaerobic genera (*Bacteroides* spp., *Lactobacillus* spp., *Parabacteroides* spp., *Clostridium* spp., etc.), 60–80% for 6 anaerobic genera (*Fusobacterium* spp., *Eggerthella* spp., *Actinobaculum* spp., *Atopobium* spp., *Anaerococcus* spp., *Flavonifracter* spp.), and lower than 60% for the other four anaerobic genera (*Eubacterium* spp., *Bilophila* spp., *Butyricimonas* spp., *Porphyromonas* spp.). Discrepancies in the correct identification rates might be due to the difficulty of obtaining satisfactory spectra from some species, such as *Mogibacterium timidum* or *Actinomyces georgiae*, and partly due to the limit of uncommon anaerobe species spectra in commercial reference libraries. Therefore, it is increasingly important to update the database of various anaerobic species, especially those lacking or poorly represented in the current version [51].

8. Summary of Possible Advantages and Limits of Microbial Identification by MALDI-TOF

As with any of the commercially available microbial identification systems, the MALDI-TOF MS has its advantages and disadvantages (Table 1). The system can identify a broad spectrum of bacteria, including Gram-positive and Gram-negative [52]. Still, Spectral interference can occur due to the presence of endospores of bacteria like Bacillus species. To overcome this, 24-h cultures should be used [53]. Pathogen fungi can be identified via MALDI-TOF mass spectra, with the contribution of processing software and spectral database of reference strain [54]. Contrary to bacteria, fungal cells are larger, and their cell wall is more rigid, so modified approaches had to be developed as regards the procedure of sample preparation, selection of a proper matrix compound, and sample deposition techniques [55]. The MALDI-TOF MS provided a high rate of species-level identifications for anaerobic isolates from clinical samples, while the rate of unidentified Gram-positive has been reduced. However, these results still pinpoint the need to include further reference spectra from this bacteria group in future reference libraries versions [56]. In some cases, because of given the accuracy of MALDI-TOF for bacterial identification, this technology is directly applied to some clinical samples, such as blood [38], urine [38], and milk [45]. The major limitation of the wide application is the number of bacteria in the blood [57] and urine [58] samples. To circumvent these difficulties, some procedures, such as the separation of blood and filtration for urine, are available. The bacterial count in the milk samples directly affects the ability of MALDI-TOF to correctly identify the bacteria [59].

Table 1. Summary of possible microbial mechanisms requiring attention during the MALDI-TOF MS examination.

Diagnostic Sample	Advantages of MALDI- TOF	Limitation of MALDI-TOF	Directions for Use
Identification of pure cultures			
$Gram^+$ bacteria	High-confidence identification [52]	Spore formation ability distorts the mass spectrum [53]	It is important to examine 24-h culture.
$Gram^-$ bacteria	High-confidence identification [52]	-	Cultures that are older than 24 h can also be examined.
Fungi	The species in the library can be reliably identified [54]	Difficult to extract the eukaryotic riboprotein [55]	An extra exploration procedure is required during sample preparation.
Anaerobic culture	High-confidence identification (If it is not spore-forming species) [56]	-	Depends on the Gram type.
Direct identification from biological samples			
Blood culture	Possible to detect bloodstream infection [38]	It is necessary for bacterial abundance above a certain threshold [57]	The separation of blood cells is required to recover the bacterial cells selectively.
Urine	Possible to detect urinary tract infections directly [38]	Above 10^3 cell.ml [58]	A membrane filtration or magnetic separation-based collection or enrichment of the pathogen.
Milk	Direct identification from samples is possible [45]	It is rare for only one microbe to be present above the detectable threshold [59]	Confirmation of the result is recommended by DNA sequencing or culturing.

9. Conclusions

The reduction of microbial-origin diseases has become a current topic. Effective medical treatment requires extensive use of rapid diagnostic methods. The MALDI-TOF technique seems to be suitable for identifying the microorganisms causing the symptoms.

However, in some cases, it has to be coupled with other analytic methods to reach sufficient results for measuring the incubation period and spread investigation. Regarding the objective of the present review, we have highlighted the benefits and some drawbacks of the MALDI-TOF application in microbial identification. The available literature data indicates that the quality of extracted proteins is highly dependent on the extraction method, in which there are significant differences. Identifying prokaryotic bacteria is easy and provides reliable results (they are closely related to the 16s-rRNA sequence outcomes), while describing eukaryotic microbes, such as yeasts and molds, often encounters obstacles. The main reason for this is the complexity of eukaryotic cells, which is reflected in the variety of ribosomal proteins. To separate them, the flight time provided by linear TOFs is sufficient only under certain conditions. Though the MALDI-TOF is normally reproducible, there are examples of variable results. For example, the different properties and individual mass spectrometer instruments, the matrix, and other solvent content, the preparation protocol, the culture conditions (medium, temperature, and the age of the colony), and the strain's biological variability. The direct detection of microbes from environmental samples by MALDI-TOF is a great opportunity for both clinical-, and veterinary diagnosis, environmental conditions, and the food industry, whereas omitting the breeding of pure cultures from samples shortens the assay time by at least 24 h. In addition, sample preparation can be automated, enabling the metadata analysis of clinical or food samples. This concept includes automatic inoculation of the sample on culture media, as well as automated cultivation and growth detection of sample cultures. The next aim is to have the colonies automatically picked and differentiated to the species level. Although MALDI-TOF MS has only recently been introduced to microbiological diagnostics, innovations and new developments show promising future applications in many cases; for example, separation techniques in typing the isolates, which would enable the acquaintance of some harmful or beneficial strains.

Author Contributions: A.H., T.K., investigation, writing—original draft preparation; Z.K., data curation; C.M.-F., formal analysis, investigation; M.R., visualization; A.H., T.K., investigation, data curation. All authors have read and agreed to the published version of the manuscript.

Funding: The study was funded by supported the European Union and co-financed by the European Social Fund (grant agreement no. EFOP-3.6.3-VEKOP-16-2017-00005) and by the European Regional Development Fund and the Hungarian Government (grant agreement no. GINOP-2.2.1-18-2020-00025).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The author declares no conflict of interest.

References

1. Rychert, J.A. Benefits, and Limitations of MALDI-TOF Mass Spectrometry for the Identification of Microorganisms. *J. Infect.* **2019**, *2*, 1–5. https://doi.org/10.29245/2689-9981/2019/4.1142.
2. Simke, F.; Fischer, P.; Marx, G.; Schweikhard, L. Simulations of a digital ion filter and a digital ion trap for heavy biomolecules. *Int. J. Mass Spectrom.* **2022**, *473*, 116779.
3. Beavis, R.C.; Chait, B.T. Cinnamic acid derivatives as matrices for ultraviolet laser desorption mass spectrometry of proteins. *Rapid Commun. Mass Spectrom.* **1989**, *3*, 432–435. https://doi.org/10.1002/rcm.1290031207. PMID: 2520223.
4. Batoy SM, A.B.; Akhmetova, E.; Miladinovic, S.; Smeal, J.; Wilkins, C.L. Developments in MALDI mass spectrometry: The quest for the perfect matrix. *Appl. Spectrosc. Rev.* **2008**, *43*, 485–550.
5. Anhalt, J.P.; Fenselau, C. Identification of bacteria using mass spectrometry. *Anal. Chem.* **1975**, *47*, 219–225.
6. Keys, C.J.; Dare, D.J.; Sutton, H.; Wells, G.; Lunt, M.; McKenna, T.; McDowall, M.; Shah, H.N. Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterisation of bacteria implicated in human infectious diseases. *Infect Genet. Evol*. **2004**, *4*, 221–242.

7. Jones, J.J.; Stump, M.J.; Fleming, R.C.; Lay, J.O.; Wilkins, C.L. Investigation of MALDI-TOF and FT-MS Techniques for Analysis of Escherichia c oli Whole Cells. *Anal. Chem.* **2003**, *7*, 1340–1347.
8. Giebel, R.; Worden, C.; Rust, S.M.; Kleinheinz, G.T.; Robbins, M.; Sandrin, T.R. Microbial fingerprinting using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) applications and challenges. *Adv. Appl. Microbiol*. **2010**, *71*, 149–184.
9. Faron, M.L.; Buchan, B.W.; Hyke, J.; Madisen, N.; Lillie, J.L.; Granato, P.A.; Wilson, D.A.; Procop, G.W.; Novak-Weekley, S.; Marlowe, E.; et al. Multicenter evaluation of the Bruker MALDI Biotyper CA system for the identification of clinical aerobic gram-negative bacterial isolates**.** *PLoS ONE* **2015**, *10*, e0141350.
10. Spanu, T.; De Carolis, E.; Fiori, B.; Sanguinetti, M.; D'Inzeo, T.; Fadda, G.; Posteraro, B. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to rpoB gene sequencing for species identification of bloodstream infection staphylococcal isolates, *Clin. Microbiol. Infect*. **2011**, *17*, 44–49.
11. Handal, N.; Bakken, J.S.; Smith, T.H.; Johnsen, B.O.; Leegaard, T.M. Anaerobic blood culture isolates in a Norwegian university hospital: Identification by MALDI-TOF MS vs 16S rRNA sequencing and antimicrobial susceptibility profiles. *Apmis* **2015**, *123*, 749–758.
12. Bizzini, A.; Greub, G. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. *Clin. Microbiol. Infect*. **2010**, *16*, 1614–1619. https://doi.org/10.1111/j.1469-0691.2010.03311.x.
13. Carlsohn, E.; Carol, L.; Nilsson, E.C. Chapter 12—Proteomic Techniques for Functional Identification of Bacterial Adhesins. In *Lectins*; Nilsson, C.L., Ed.; Elsevier Science B.V.: Amsterdam, The Netherlands, 2007; pp. 299–325.
14. Wieser, A.; Schneider, L.; Jung, J.; Schubert, S. MALDI-TOF MS in microbiological diagnostics—Identification of microorganisms and beyond (mini review). *Appl. Microbiol. Biotechnol.* **2012**, *93*, 965–974.
15. Croxatto, A.; Prod'hom, G.; Greub, G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology, *FEMS Microbiol. Rev.* **2012**, *36*, 380–407. https://doi.org/10.1111/j.1574-6976.2011.00298.x.
16. Camara, J.E.; Hays, F.A. Discrimination between wild-type and ampicillin-resistant Escherichia coli by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Bioanal. Chem*. **2007**, 389:1633–1638.
17. Edwards-Jones, V.; Claydon, M.A.; Evason, D.J.; Walker, J.; Fox, A.J.; Gordon, D.B. Rapid discrimination between methicillin-sensitive and methicillin-resistant Staphylococcus aureus by intact cell mass spectrometry. *J. Med. Microbiol*. **2000**, *49*, 295–300.
18. Ryzhov, V.; Hathout, Y.; Fenselau, C. Rapid characterization of spores of Bacillus cereus group bacteria by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. *Appl. Environ. Microbiol*. **2000**, *66*, 3828–3834.
19. Seng, P.; Drancourt, M.; Gouriet, F.; La Scola, B.; Fournier, P.E.; Rolain, J.M.; Raoult, D. Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis*. **2009**, *49*, 543–551.
20. Eigner, U.; Holfelder, M.; Oberdorfer, K.; Betz-Wild, U.; Bertsch, D.; Fahr, A.M. Performance of a matrix-assisted laser desorption ionizationtime- of-flight mass spectrometry system for the identification of bacterial isolates in the clinical routine laboratory. *Clin. Lab*. **2009**, *55*, 289–296.
21. Cherkaoui, A.; Hibbs, J.; Emonet, S.; Tangomo, M.; Girard, M.; Francois, P.; Schrenzel, J. Comparison of two matrixassisted laser desorption ionization–time of flight mass spectrometry methods with conventional phenotypic identification for routine bacterialnspeciation. *J. Clin. Microbiol*. **2010**, *48*, 1169–1175.
22. Kollef, M.H. Inadequate antimicrobial treatment: An important determinant of outcome for hospitalized patients. *Clin. Infect. Dis.* **2000**, *4*, 131–138.
23. Leibovici, L.; Shraga, I.; Drucker, M.; Konigsberger, H.; Samra, Z.; Pitlik, S.D. The benefit of appropriate empirical antibiotic treatment in patients with bloodstream infection. *J. Intern. Med*. **1998**, *244*, 379–386.
24. Zhou, W.; Petricoin, E.F.; Longo, C. Mass Spectrometry-Based Biomarker Discovery. *Methods Mol. Biol*. **2017**, *1606*, 297–311.
25. Florio, W.; Morici, P.; Ghelardi, E.; Barnini, S.; Lupetti, A. Recent advances in the microbiological diagnosis of bloodstream infections. *Crit. Rev. Microbiol*. **2018**, *44*, 351–370.
26. Dubourg, G.; Raoult, D.; Fenollar, F. Emerging methodologies for pathogen identification in bloodstream infections: An update. *Expert Rev. Mol. Diagn*. **2019**, *19*, 161–173.
27. Faron, M.L.; Buchan, B.W.; Ledeboer, N.A. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for use with positive blood cultures: Methodology, performance, and optimization. *J. Clin. Microbiol*. **2017**, *55*, 3328–3338.
28. Prod'hom, G.; Bizzini, A.; Durussel, C.; Bille, J.; Greub, G. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for direct bacterial identification frompositive blood culture pellets. *J. Clin. Microbiol*. **2010**, *48*, 1481–1483.
29. Ferreira, L.; Sánchez-Juanes, F.; Porras-Guerra, I.; García-García, M.I.; García-Sánchez, J.E.; González-Buitrago, J.M.; Muñoz-Bellido, J.L. Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clin. Microbiol. Infect*. **2011**, *17*, 546–551.
30. Moussaoui, W.; Jaulhac, B.; Ho_mann, A.M.; Ludes, B.; Kostrzewa, M.; Riegel, P.; Prévost, G. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry identifies 90% of bacteria directly from blood culture vials. *Clin. Microbiol. Infect*. **2010**, *16*, 1631–1638.
31. Yonetani, S.; Ohnishi, H.; Ohkusu, K.; Matsumoto, T.; Watanabe, T. Direct identification of microorganisms from positive blood cultures by MALDI-TOF MS using an in-house saponin method. *Int. J. Infect. Dis*. **2016**, 52, 37– 42.

32. Schubert, S.; Weinert, K.; Wagner, C.; Gunzl, B.; Wieser, A.; Maier, T.; Kostrzewa, M. Novel, improved sample preparation for rapid, direct identification from positive blood cultures using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. *J. Mol. Diagn*. **2011**, *13*, 701–706.
33. Fothergill, A.; Kasinathan, V.; Hyman, J.; Walsh, J.; Drake, T.; Wang, Y.F. Rapid identification of bacteria and yeasts from positive-blood-culture bottles by using a lysis-filtration method and matrix-assisted laser desorption ionization-time of flight mass spectrum analysis with the SARAMIS database. *J. Clin. Microbiol*. **2013**, *51*, 805–809.
34. Ashizawa, K.; Murata, S.; Terada, T.; Ito, D.; Bunya, M.; Watanabe, K.; Teruuchi, Y.; Tsuchida, S.; Satoh, M.; Nishimura, M.; et al. Applications of copolymer for rapid identification of bacteria in blood culture broths using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *J. Microbiol. Methods* **2017**, *139*, 54–60.
35. Yonezawa, T.; Watari, T.; Ashizawa, K.; Hanada, D.; Yanagiya, T.; Watanabe, N.; Terada, T.; Tomoda, Y.; Fujii, S. Dvelopment of an improved rapid BACpro® protocol and a method for direct identification from blood-culture-positive bottles using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *J. Microbiol. Methods* **2018**, *148*, 138–144.
36. Oviaño, M.; Bou, G. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for the Rapid Detection of Antimicrobial Resistance Mechanisms and Beyond. *Clin. Microbiol. Rev*. **2018**, *32*, e00037-18.
37. Miller, M.B.; Atrzadeh, F.; Burnham, C.A.; Cavalieri, S.; Dunn, J.; Jones, S.; Mathews, C.; McNult, P.; Meduri, J.; Newhouse, C.; et al. ASM Clinical and Public Health Microbiology Committee and the ASM Corporate Council. Clinical Utility of Advanced Microbiology Testing Tools. *J. Clin. Microbiol*. **2019**, *57*, e00495-19.
38. Croxatto, A.; Prod'hom, G.; Faverjon, F.; Rochais, Y.; Greub, G. Laboratory automation in clinical bacteriology: What system to choose? *Clin. Microbiol. Infect*. **2016**, *22*, 217–235.
39. Maier, T.; Schwarz, G.; Kostrzewa, M.; Fahr, A.; HolfelderR, M.; Eigner, U.; Weller, U. Rapid identification of bacteria from blood cultures using MALDI-TOF MS. In Proceedings of the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, DC, USA, 25–28 October 2008**.**
40. Ferreira, L.; Sánchez-Juanes, F.; González-Ávila, M.; Cembrero-Fuciños, D.; Herrero-Hernández, A.; González-Buitrago, J.M.; Muñoz-Bellido, J.L. Direct identification of urinary tract pathogens from urine samples by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol*. **2010**, *48*, 2110–2115.
41. Guo, Z.; Liu, Y.; Li, S.; Yang, Z. Interaction of bacteria and ion-exchange particles and its potential in separation for matrix-assisted laser desorption/ionization mass spectrometric identification of bacteria in water. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3983–3993.
42. Íñigo, M.; Coello, A.; Fernández-Rivas, G.; Rivaya, B.; Hidalgo, J.; Quesada, M.D.; Ausina, V. Direct identification of urinary tract pathogens from urine samples, combining urine screening methods and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol*. **2016**, *54*, 988–993.
43. Veron, L.; Mailler, S.; Girard, V.; Muller, B.H.; L'Hostis, G.; Ducruix, C.; Lesenne, A.; Richez, A.; Rostaing, H.; Lanet, V.; et al. Rapid urine preparation prior to identification of uropathogens by MALDI-TOF MS. Eur. *J. Clin. Microbiol. Infect. Dis.* **2015**, *34*, 1787–1795.
44. Schwarz, G.; Maier, T.; Kostrzewa, M.; Boogen, C.; Weller, U. Rapid identification of bacteria causing urinary tract infections by MALDI-TOF. In Proceedings of the Abstract of the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) and the 46th Infectious Diseases Society of America (IDSA), Washington, DC, USA, October 25-28, 2008.
45. Wilson, D.J.; Middleton, J.R.; Adkins, P.R.F.; Goodell, G.M. Test agreement among biochemical methods, matrix-assisted laser desorption ionization–time of flight mass spectrometry, and 16S rRNA sequencing for identification of microorganisms isolated from bovine milk. *J. Clin. Microbiol*. **2019**, *57*, e01381-18. https://doi.org/10.1128/JCM.01381-18.
46. Pukančíková, L.; Lipničanová, S.; Kačániová, M.; Chmelová, D.; Ondrejovič, M. Natural microflora of raw cow milk and their enzymatic spoilage potential. *Nova Biotech Et Chim* **2016**, *15*, 142–155.
47. Norin, E. How normal is a "normal" flora in animal or man? *Anaerobe* **2011**, *17*, 431–432.
48. Kim, J.; Lee, Y.; Park, Y.; Kim, M.; Choi, J.Y.; Yong, D.; Jeong, S.H.; Lee, K. Anaerobic bacteremia: Impact of inappropriate therapy on mortality. *Infect. Chemother*. **2016**, *48*, 91–98.
49. Lau, S.K.; Tang, B.S.; Teng, J.L.; Chan, T.M.; Curreem, S.O.; Fan, R.Y.; Ng, R.H.; Chan, J.F.; Yuen, K.Y.; Woo, P.C. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for identification of clinically significant bacteria that are difficult to identify in clinical laboratories. *J. Clin. Pathol*. **2014**, *67*, 361–366.
50. Marinach-Patrice, C.; Lethuillier, A.; Marly, A.; Brossas, J.-Y.; Gené, J.; Symoens, F.; Datry, A.; Guarro, J.; Mazier, D.; Hennequin, C. Use of mass spectrometry to identify clinical Fusarium isolates. *Clin. Microbiol. Infect*. **2010**, *15*, 634–642.
51. Lavigne, J.P.; Espinal, P.; Dunyach-Remy, C.; Messad, N.; Pantel, A.; Sotto, A. Mass spectrometry: A revolution in clinical microbiology? *Clin. Chem. Lab. Med*. **2013**, *51*, 257–270.
52. Biswas, S.; Rolain, J.M. Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. *J. Microbiol. Methods* **2013**, *92*, 14–24.
53. Clark, A.E.; Kaleta, E.J.; Arora, A.; Wolk, D.M. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev*. **2013**, *26*, 547–603.
54. Chalupová, J.; Raus, M.; Sedlářová, M.; Šebela, M. Identification of fungal microorganisms by MALDI-TOF mass spectrometry, *Biotechnol. Adv.* **2014**, *32*, 230–241, ISSN 0734-9750.
55. Carlile, M.J.; Gooday, G.W.; Watkinson, S.C. *The Fungi*, 2nd ed.; Academic Press Ltd.: San Diego, CA, USA, 2001.

56. Alcala, L.; Marı´n M.; Ruiz, A.; Quiroga, L.; Zamora-Cintas, M.; Ferna´ ndez-Chico, M.A.; Muñoz, P. and Rodrı´guez-Sa´ nchez, B. Identifying Anaerobic Bacteria Using MALDI-TOF Mass Spectrometry: A Four-Year Experience. *Front. Cell. Infect. Microbiol*. **2021**, *11*, 521014. https://doi.org/10.3389/fcimb.2021.521014.
57. Tsuchida, S.; Umemura, H.; Nakayama, T. Current Status of Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in Clinical Diagnostic Microbiology. *Molecules* **2020**, *25*, 4775. https://doi.org/10.3390/molecules25204775. PMID: 33080897; PMCID: PMC7587594.
58. Kim, Y.; Park, K.G.; Lee, K.; Park, Y.J. Direct identification of urinary tract pathogens from urine samples using the Vitek MS system based on matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Ann. Lab. Med.* **2015**, *35*, 416–422. https://doi.org/10.3343/alm.2015.35.4.416.
59. Barreiro, R.J.; Gonçalves, L.J.; Braga, C.A.P.; Dibbern, G.A.; Eberlin, N.M.; dos Santos, V.M. Non-culture-based identification of mastitis-causing bacteria by MALDI-TOF mass spectrometry. *J. Dairy Sci.* **2017**, 100, 2928–2934, ISSN 0022-0302, https://doi.org/10.3168/jds.2016-11741.