



Mass spectrometry in research laboratories and clinical diagnostic: a new era in medical mycology

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Abstract

Diagnosis by clinical mycology laboratory plays a critical role in patient care by providing definitive knowledge of the cause of infection and antimicrobial susceptibility data to physicians. Rapid diagnostic methods are likely to improve patient. Aggressive resuscitation bundles, adequate source control, and appropriate antibiotic therapy are cornerstones for success in the treatment of patients. Routine methods for identifying clinical specimen fungal pathogen are based on the cultivation on different media with the subsequent examination of its phenotypic characteristics comprising a combination of microscopic and colony morphologies. As some fungi cannot be readily identified using these methods, molecular diagnostic methods may be required. These methods are fast, but it can cost a lot. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is suitable for high-throughput and rapid diagnostics at low costs. It can be considered an alternative for conventional biochemical and molecular identification systems in a microbiological laboratory. The reliability and accuracy of this method have been scrutinized in many surveys and have been compared with several methods including sequencing and molecular methods. According to these findings, the reliability and accuracy of this method are very high and can be trusted. With all the benefits of this technique, the libraries of MALDI-TOF MS need to be strengthened to enhance its performance. This review provides an overview of the most recent research literature that has investigated the applications and usage of MT-MS to the identification of microorganisms, mycotoxins, antifungal susceptibility examination, and mycobiome research.

Keywords MALDI-TOF · Pathogenic fungi · Diagnosis · Identification · Mycobiome

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Introduction

Considering the significant increase in fungal infections within the recent decades, it is essential to use more rapid and accurate methods to identify the causative agents which is helpful for the correct and prompt diagnosis and successful treatment of fungal infections [1, 2]. However, the emerging yeast and mold pathogens have proven to be challenging with regard to accurate identification of the pathogen in clinical laboratories that classically use microscopic examinations and biochemical tests for diagnosis [3]. Although the classic methods based on phenotypic characteristics play an important role in identifying pathogenic fungi, these methods have several drawbacks, including non-specificity and time-consuming; moreover, the appearance of physiological and morphological characteristics in traditional methods is influenced by many factors such as growth rate, the presence of a proper amount of organism in the clinical specimen, and previous treatments [4, 5]. Additionally, conventional methods require developing a laboratory expert system that is associated with a high cost of testing and a high rate of false-negative, while eventually, a significant number of fungal strains remain uncertain [5]. The use of molecular techniques has provided sensitive and more specific methods for scientists, researchers, and laboratory experts to easily identify different types of microorganisms such as pathogenic fungi with a higher degree of reliability [6]. Among the molecular methods, sequencing is marked as the golden standard for the accurate identification of the species level of fungi; however, sequencing is expensive and relatively time-consuming [7]. Recently, the matrix-assisted laser

desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been used for rapid and accurate identification of fungi frequently [8, 9]. The MALDI-TOF MS is a substitute for conventional and DNA-dependent methods for the identification of fungi and bacteria [10]. This method is based on the detection of protein with a molecular weight ranging between 2 and 20 kDa, as bio-taxonomy markers [11]. The remarkable advantages of the MALDI-TOF MS method over genetic and morphological methods are simplicity and short time for sample preparation and analysis. The testing and analysis, including sample preparation and evaluation of data, can be done within a few minutes (Fig. 1) [11]. Methods for microbial detection should be simple, low-cost, rapid, and accurate. Also, fast and reliable means of identification of bacteria and fungi are prerequisites for effective treatment. In all cases mentioned above, the MALDI-TOF MS technique is appropriate for microbial identification especially in identifying yeasts and molds [11]. Currently, the advent of the MT-MS has made a great deal of change in the detection and identification of the disease causative agents in microbiology laboratories [12]. The identification of organisms by the MALDI-TOF MS method is based on characteristic protein patterns derived from the microbes and comparison of this unique patterns with the library of spectra database on the device system [12, 13]. In fact, the most important point in the success of the MALDI-TOF MS system to identify the organisms to species level is library database [14]. This method has been used to identify fungi, including molds, yeasts such as *Candida* and *Cryptococcus*, and dermatophytes [14–16]. The identification of molds by the MT-MS technique is challenging, and

Fig. 1 MALDI-TOF mass spectrometry and its applications in species identification, antifungal drug resistance assay, and screening of mycotoxins

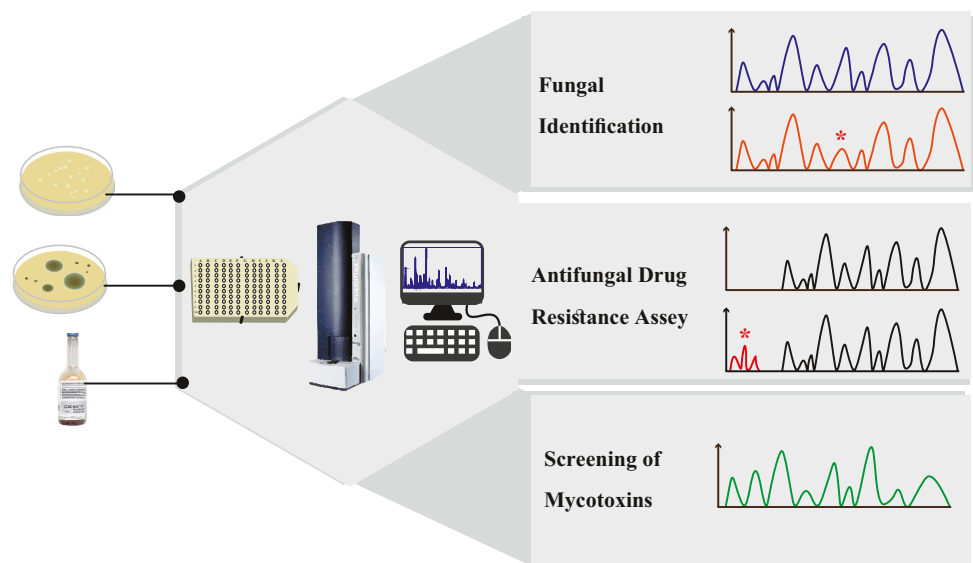


Table 1 Overview of MALDI-TOF mass spectrometry in the identification of pathogenic fungi

No	Author, year	Country	No. of isolates	Correct identification	MALDI-TOF system	Organism
Molds						
1	Hettick et al. 2011 [18]	USA	12	100%	MALDI-TOF	<i>Aspergillus</i> sp.
2	Buskirk et al. 2011 [19]	USA	ND	Suppression by Melanie	MALDI (BioRad)	<i>A. niger</i>
4	De Carolis et al. 2012 [20]	Italy	94	96.8%	MALDI Biotyper 2.0	<i>Aspergillus</i> , <i>Fusarium</i> , and <i>Mucorales</i>
5	Bille et al. 2012 [21]	France	64	98.4%	Andromas software	<i>Aspergillus</i> sp.
6	Iriart et al. 2012 [22]	France	36	93.2%	VITEK MS	<i>Aspergillus</i> sp.
7	Lau et al. 2013 [23]	USA	421	88.9%	Biotyper	Molds
9	Ranque et al. 2014 [24]	France	625	89%	Bruker	Molds
10	Tam et al. 2014 [25]	Honk Kong	11	33.3% Saramis, 66.6% VITEK	VITEK MS & Saramis	<i>Aspergillus</i> sp.
11	Lopes et al. 2014 [26]	Brazil	51	63.5–99.5% based on media used	Biotyper 1.0	<i>Metarhizium anisopliae</i> complex
13	Panda et al. 2014 [27]	India	37	86.4% molds	Bruker	Molds
14	Kondori et al. 2015 [28]	Sweden	51	> 70%	Saramis	Black molds
15	Gruenwald et al. 2015 [29]	Germany	20	100%	Bruker	<i>Stachybotrys</i> sp.
16	Masih et al. 2016 [30]	India	45	95%	Bruker database	Rare <i>Aspergillus</i> sp.
17	McMullen et al. 2016 [31]	USA	319	VITEK (66.8%), Saramis (76.8%)	VITEK MS & Saramis	Molds
18	Sleiman et al. 2016 [32]	Australia	117	<i>Aspergillus</i> , 93%; <i>Fusarium</i> , 84%; <i>Scedosporium</i> , 94%	VITEK MS	<i>Aspergillus</i> , <i>Fusarium</i> , <i>Scedosporium</i>
19	Atalay et al. 2016 [33]	Turkey	23	91.7–100%	VITEK MS	<i>Aspergillus</i> sp.
20	Borman et al. 2017 [34]	UK	89	100%	MALDI Biotyper (version 3.0)	Black fungi
21	Singh et al. 2017 [35]	India	72	100%	Bruker Daltonics	Black molds
22	Huang et al. 2017 [36]	China	153	98.7%	Biotyper 3.1 and V1.0	Molds
23	Park et al. 2017 [37]	Korea	345	94.5–96%	MALDI Biotyper 1.0	<i>Aspergillus</i> sp.
24	Fraser et al. 2017 [38]	UK	57	100%	Bruker	Black molds
25	Normand et al. 2017 [39]	France	510	87.35%	MALDI Biotyper	Molds
26	Valero et al. 2018 [40]	Spain	63	91.2%	MALDI Biotyper 4.0	<i>Histoplasma capsulatum</i>
27	Shao et al. 2018 [41]	China	111	100% genus level, 81.1% up to species level	Bruker MALDI Biotyper 1.0	Mucorales
28	Rychert et al. 2018 [42]	USA	1601	98%	VITEK MS v3.0	Molds

Table 1 (continued)

No	Author, year	Country	No. of isolates	Correct identification	MALDI-TOF system	Organism
29	Hedayati et al. 2019 [16]	Iran	200	88%	Bruker	<i>A. flavus</i> and <i>A. oryzae</i>
30	Américo et al. 2019 [43]	Brazil	106	84.7%	VITEK MS	<i>Aspergillus</i> sp.
31	Reeve et al. 2019 [44]	England	5	80%	Bruker	<i>Penicillium</i> sp.
32	Peng et al. 2019 [45]	China	123	92.7%	Bruker	Molds
33	Sacheli et al. 2020 [46]	Belgium	129	71.4%	Bruker	Molds and dermatophytes
34	Robert et al. 2020 [47]	France	74	93.9%	Bruker	Molds
35	Gómez-Velásquez et al. 2020 [48]	Colombia	62	88.7%	in-House	Molds
36	Martin et al. 2020 [49]	France	80	68%	Bruker	Molds
37	Lee et al. 2020 [50]	Korea	505	86.5%	ASTA	Molds
Dermatophytes						
30	Erhard et al. 2008 [51]	Germany	20	99.9%	Axima@Saramis	5 different dermatophytes
31	Theel et al. 2011 [52]	Minnesota, USA	192	4.7%, 36.8%, 59.6%	MALDI Biotyper	7 different dermatophytes
32	Alshawa et al. 2012 [53]	France	381	91.9%	Andromas	8 different dermatophytes
33	De Respini et al. 2013 [54]	Switzerland	141	95.8%	Saramis	9 different dermatophytes
34	L'Ollivier et al. 2013 [55]	France	133	97.8%	MALDI Biotyper	17 different dermatophytes
35	Nenoff et al. 2013 [56]	Germany	285	99.3%	Axima@Saramis	21 different dermatophytes
36	De Respini et al. 2013 [54]	Switzerland	141	95.8%	Axima@Saramis	9 different dermatophytes
37	Packeu et al. 2014 [57]	Belgium	176	40–100%	MALDI Biotyper	7 different dermatophytes
38	De Respini et al. 2014 [58]	Switzerland	134	98.4%	VITEK MS and Axima@Saramis	17 different dermatophytes
39	Calderaro et al. 2014 [59]	Italy	64	ND	Bruker	11 different dermatophytes
40	Karabıcak et al. 2015 [60]	Turkey	115	13.5%	Bruker	5 different dermatophytes
41	Hedayati et al. 2019 [15]	Iran	94	47%	Bruker	5 different dermatophytes
42	Gnat et al. 2020 [61]	Poland	41	97.2%	Bruker	6 different dermatophytes
43	Shao et al. 2020 [62]	China	113	90.1%	Bruker	4 different dermatophytes
Yeast						
42	Mancini et al. 2013 [63]	Italy	197	Bruker (89.8%) VITEK MS (100%)	VITEK MS & Bruker Biotyper (v 3.1)	10 genera and 44 species
43	Lohmann et al. 2013 [64]	France	312	Biotyper (87.2%)		

Table 1 (continued)

No	Author, year	Country	No. of isolates	Correct identification	MALDI-TOF system	Organism
Saramis (82.7%)	Biflex III-Biotyper & Axima@ Saramis	8 genera and 45 species				
44	Pence et al. 2014 [65]	USA	117	VITEK (96.6%)		
Bruker (88.0%)	VITEK MS & Bruker Biotyper (v 3.1)	6 genera and 18 species				
45	Taj-Aldeen et al. 2014 [66]	Qatar	201	100%	Bruker	<i>Candida</i> spp.
46	Kathuria et al. 2015 [67]	Netherland	107	100%	MALDI Biotyper 3.1	<i>Candida</i> spp.
47	Vatanshenassan et al. 2019 [68]	Netherlands	50	100%	Bruker	<i>C. auris</i>
48	Ceballos-Garzon et al. 2020 [69]	Colombia	300	100%	In-house	<i>C. auris</i>
49	Noni et al. 2020 [70]	Greece	33	100%	Bruker	Yeast
50	Zvezdanova et al. 2020 [71]	Spain	70	96.55%	Bruker	<i>Cryptococcus</i> sp.

several factors might have influence on the output of the system, including the presence or absence of conidia, the colony growth stages, the presence of melanin, and the preparation protocol [17]. This review introduces how MALDI-TOF MS can be used in fungal identification, mycotoxin identification, antifungal drug resistance assay and its superior efficacy, and potential of its future applications (Table 1) [15, 16, 18–71].

MALDI-TOF MS in fungal identification

Identification of yeast

Despite therapeutic advances, fungal infections caused by yeasts remain widespread. Most frequently of yeast infections are caused by *Candida*, *Cryptococcus*, and *Malassezia* and may occur as severe opportunistic infections [72, 73]. Several studies have shown that many yeasts have been identified by the MALDI-TOF MS system [9, 70, 74–77]. The quality of database is the main pillars of microbial identification by MT-MS. Both the VITEK MS and the MALDI Biotyper have massive databases. VITEK MS IVD v2.0 database of BioMerieux includes 193 several of yeast species, including *Candida*, *Malassezia*, and *Cryptococcus*, as well as several bacteria family. In addition, Bruker MALDI Biotyper can be used to detect more than 210 species, including gram-negative and gram-positive bacteria and yeasts [4, 78, 79].

Candida species

Candida albicans *Candida* is a known prominent causative agent of bloodstream infection that is usually associated with a high mortality rate ranging between 30.4 and 53.6%, especially among immunocompromised patients and those with the acute disease. This opportunistic fungal pathogen is the most prevalent cause of fungal yeast infections in human [74, 75, 80–83]. The rapid and precise identification of this fungi can significantly improve patient care [83].

Significant numbers of studies using MALDI-TOF MS for the rapid identification of *C. albicans* have been published (87.2–100%) [74, 75, 80, 82, 83]. MALDI-TOF MS analysis with the direct formic acid (FA) sample processing method was expected to reliably identify the closely related species of *C. dubliniensis* and *C. albicans* [82]. In Sendin et al. study on mouse models of *C. albicans* colonization and infection, the MT-MS has revealed the specific signal for tissue invasion, recommending that clinical estimation of its efficiency to differentiate infected and colonized patients to be promising [83].

Candida glabrata *Candida glabrata* is one of the dominant causes of fungal infection in humans, after *C. albicans*, particularly in immunocompromised hosts [84, 85]. Using MALDI-TOF MS for *C. glabrata* identification would be a cost-effective and easy-to-use alternative to sequencing and other classical DNA-based typing methods [86, 87].

In 2018, Maldonado et al. have compared conventional and MALDI-TOF MS methods for *C. glabrata* identification. The direct concordance between the conventional identification method and MALDI-TOF MS was 92.5% [88]. Furthermore, Gorton et al. have reported *C. nivariensis* from a renal transplant patient with continuous candiduria. Biochemical profiling misidentified isolates as *C. glabrata* and *C. inconspicua*. The MALDI-TOF MS analysis and the sequencing of ITS regions identified all isolates as *C. nivariensis* [89].

Candida tropicalis Non-*albicans* *Candida* species, including *C. tropicalis* are now increasingly identified as human pathogens. The remarkable emergence of these species as a frequent cause of fungal infection can be related to improved identification methods and also attributed to the increased patient's survival rate, the interventions, and various therapy [84, 90]. Wang et al. in 2017 via using MALDI-TOF MS and VITEK MS systems have identified genetically confirmed blood isolates of *C. tropicalis* [90].

MT-MS significantly increases the detection capacities of the phenotypic procedure by decreasing the stagnation of results and giving precise identification of *C. tropicalis*. Therefore, this method is cost-effective and should be performed mainly in resource-poor states [91].

Candida parapsilosis *Candida parapsilosis* is known as an opportunistic pathogen related to nosocomial infections. Human-made environments with high humidity, such as water distribution systems and dishwashers, could be leading sources of *C. parapsilosis* [92].

In several studies, MALDI-TOF MS was shown to be a reliable procedure for the identification of *C. parapsilosis* [93–96]. Quiles-Melero et al. evaluated the MALDI-TOF MS VITEK MS™ system for the identification of *C. parapsilosis* successfully [93]. However, Mynáriková et al. investigation illustrated that the detection of biofilm formation ability in *C. parapsilosis* sensu stricto strains by MALDI-TOF MS was impossible [95]. MALDI-TOF MS method represents a hopeful alternative for regular identification of yeast species, including commonly and seldom clinically encountered yeast isolates and multiple species belonging to *C. parapsilosis* complex [94].

Candida auris As an emergent fungal pathogen, infections caused by *C. auris* are commonly nosocomial, bloodstream-infecting, and multi-resistant, which can be associated with high mortality rates. *C. auris* is a recently discovered yeast that has been reported and documented from Asian countries and South Africa, posing serious global health threat [97]. Accordingly, early diagnosis of *C. auris* is necessary for proper treatment and prevention of drug resistance and reducing the other life-threatening factors [98]. It has been proven that MALDI-TOF MS is stronger and more rapid

method for the identification of *C. auris* [67, 69]. Kathuria et al. in 2015 evaluated *C. auris* prevalence among 102 clinical isolates formerly reported as *C. haemulonii* or *C. famata* by the VITEK 2 system. A fraction of 88.2% of the isolates were reliably confirmed as *C. auris* using MALDI-TOF MS [67]. *C. auris* is usually still neglected in the microbiology laboratories, as approximately 90% of the isolates were mistakenly identified by the routine identification methods as *C. haemulonii* [67]. On the other hand, Prakash et al. studied about 104 global *C. auris* from different countries. This study reveals that although MALDI-TOF MS has relatively adequate spectral profiling and indications of geographical clustering, it did not match completely with the genotyping methods (e.g., sequence typing (MLST) and amplified fragment length polymorphism (AFLP) fingerprinting) [99]. It is noteworthy that in 2018, Sterkel et al. reported that upgrading the safety of laboratory technicians without reduction in the quality of results is attainable by using quick-tube extraction method for the MALDI-TOF MS analysis of *Candida* spp. and *C. auris* [100].

Trichosporon species

Trichosporon species are the normal human mycobiota of the skin and gastrointestinal tract. This ubiquitous fungus usually causes superficial infections especially in immunocompromised patients [101]. High mortality rates are attributed to *Trichosporon* profound infections in immunocompromised individuals, and hence, rapid and accurate identification ensues accelerated discovery of targeted antifungal therapy [102].

However, until recently, MALDI-TOF MS has been less used for the basidiomycetous fungi. It is currently a noteworthy method for the identification of *Trichosporon* isolates at the species level [102]. Analysis of positive blood cultures infected by *T. asahii* had quick and accurate identification by mass spectra, obtained by VITEK MS™ system [103]. Despite the appropriate efficiency for the identification of some *Trichosporon* species, Almeida et al. have shown that the present IVD database from the VITEK MS requires an upgrade for better identification [102]. They found a similarity outcome between MALDI-TOF MS and regular identification (RI) among 91.5% of isolates. Molecular identification methods showed 98.6% isolates were identified accurately by MALDI-TOF MS. It revealed the potential of MALDI-TOF MS-based identification as a valid, fast, and cost-effective alternative to RI for *Trichosporon* sp. [102]. The MALDI-TOF MS results were matched to the results obtained by ITS sequence analysis in 94% cases. Misidentifications of non-*Trichosporon* species is the main reason of this lower identification rate [64]. MALDI-TOF MS could replace past phenotypic methods for *Trichosporon* identification in clinical laboratories. Developing the mass

spectrum database by adding the number of reference strains for closely related species, including those of nonclinical sources, could boost the efficiency of MALDI-TOF MS-based for optimal species identification performance of those arthroconidial fungi in medical laboratories [103].

***Geotrichum* species**

The pathogenicity of the *G. candidum* in humans has not been clearly determined. The emergence of this opportunistic fungi is uncommon and has been reported solely in patients with deep immunodeficiency, especially in patients with hematological malignancies [76]. Miglietta et al. have identified *G. capitatum* isolated from the blood of a 78-year-old male treated with salvage therapy after acute myeloid leukemia (AML) relapse by using VITEK 2 and MALDI-TOF MS [104]. The performances of the Bruker Daltonics and the Shimadzu systems for yeast identification were satisfactory and comparable under routine clinical conditions, even though they were different considering the sample preparation, database content, and spectrum analysis [105]. Several investigations revealed that MALDI-TOF MS could be a fast and reliable method for identifying *Geotrichum*, providing easy explanation of results and meanwhile requiring the lowest expenditure of consumables [104–107].

***Cryptococcus* species**

The genus *Cryptococcus* consists of pathogenic encapsulated yeasts causing an invasive mycosis. *Cryptococcus* sp. could cause widespread disease outbreaks in susceptible populations including HIV-positive patients [108]. Cryptococcosis has two main etiologic agents, *C. neoformans* and *C. gattii*, belonging to phylum Basidiomycota. They can be differentiated from other pathogenic yeasts such as *Candida* based on the presence polysaccharide capsule, production of melanin, and urease [108].

MALDI-TOF MS analyzer is practical for *Cryptococcus* species identification [71, 109]. Tarumoto et al. have used both MALDI-TOF MS and genetic sequencing for the identification of 8 cases; the retained *Cryptococcus* species. *C. neoformans* was the pathogen identified in all 8 cases using MALDI-TOF MS, yet genetic sequencing identified one of these as *C. curvatus* [109]. MALDI-TOF MS facilely distinguishes the newly recognized species including *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* as distinct species and also five species within *C. gattii* as revealed by Hagen et al. [110]. Bruker Biotyper MALDI-TOF MS was performed for species identification of *Cryptococcus* in other study [111]. The possibility of creating and transferring an in-house library adds to the expediency of MALDI-TOF MS as an important tool for the fast and low-cost identification of *Cryptococcus* species [112]. The

identification rate of common and uncommon yeast isolates in simple direct on-plate FA extraction/Biotyper method is close to VITEK MS methods, but “in-tube formic acid/acetonitrile extraction” is required for *C. neoformans* identification by Biotyper [113]. MALDI-TOF MS is a rapid identification method for the accurate diagnosis of the two currently identified human pathogenic *Cryptococcus* species, offering a simple method for the segregation of the eight major molecular types and the detection of hybrid strains within this species complex [114]. Compared to DNA sequence analysis, MALDI-TOF MS correctly identified 100% of *Cryptococcus* species, including pathogens *C. neoformans* and *C. gattii* [115]. Zevezdanova et al. have demonstrated 96.55% of *Cryptococcus* isolates tested were perfectly identified regarding their molecular type designations [71]. Siqueira et al. have explained that an in-house database provided accurate identification for all *C. neoformans* and *C. gattii* genotypes; due to the inter-genotypic mass spectral resemblance, a stringent post-analytic evaluation is obligatory to provide correct genotype identification [114].

Thomaz et al. have investigated the effect of the capsule size for *C. neoformans* and *C. gattii* identification at the species level by Bruker MALDI-TOF MS. After experimental capsule size modulation, they have reported that lessening the capsule size prompted ameliorated identification by Bruker MALDI-TOF MS among all of the reference strains analyzed [112]. MALDI-TOF MS could be a feasible supersede to regular techniques not only for the identification of fungal isolates, but also for the differentiation of clinical fungal isolates including *Cryptococcus* species. However, the major constraint of this technique is that MS identification could be more precise only if the reference spectrum of the fungal species is obtainable in the database [116].

***Malassezia* species**

Malassezia is a lipophilic yeast that exists on the skin of humans and warm-blooded animals as commensal. *Malassezia* species are involved in some skin diseases such as pityriasis versicolor, seborrheic dermatitis, catheter-related infections, and infections in infants treated with fat emulsions [117, 118]. Microscopic examination, culture, and lipid requirement test are classic methods of diagnosing *Malassezia*, but due to similarity in morphology and nutritional requirements, they are not able to accurately detect different species of *Malassezia* [117]. Molecular methods such as PCR and gene sequencing can differentiate *Malassezia* species. The MALDI-TOF MS can identify similar *Malassezia* species using the direct formic acid method [117]. In a study by Kolecka et al. using the MALDI-TOF MS technique, 14 reference *Malassezia* species were studied and identified. According to the confirmation of the results

by gene sequencing, a database was created to identify clinical specimens of *Malassezia*. Then, 206 clinical specimens were examined using the MALDI-TOF MS technique, in which different species of *Malassezia* were identified [119]. In another study, 39 culture-positive pityriasis versicolor specimens were examined by MALDI-TOF MS, of which 100% were *Malassezia furfur* [120]. Various studies have demonstrated that the MALDI-TOF MS technique for detecting *Malassezia* species has good accuracy and sensitivity. *Malassezia* strains have been detected by MALDI-TOF MS which include *M. furfur*, *M. globosa*, *M. caprae*, *M. cuniculi*, *M. dermatitis*, *M. equina*, *M. japonica*, *M. nana*, *M. obtusa*, *M. pachydermatis*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, and *M. yamatoensis* [117–120].

Identification of molds

Aspergillus species

Invasive mold infections are life-threatening, occurring among immunocompromised patients, especially in recipients of hematopoietic stem cell or solid-organ transplantation [30], resulting an increase in the cost of treatment [39]. Several studies have shown good ability of MALDI-TOF MS for accurate identification of molds [44–50, 121]. Among filamentous fungi, *Aspergillus* is the most common causative agent of invasive mold infections [122]. MALDI-TOF MS may be a reasonable replacement to DNA sequence-based identification of common *Aspergillus* species as well as uncommon, cryptic, or morphologically undistinguishable species [16, 122]. Nevertheless, in Tam et al. study, Saramis (VITEK MS research-use-only [RUO] system) and VITEK MS (VITEK MS in vitro device [IVD] system) databases were utilized to identify *A. flavus* strains (8 clinical isolates and 1 reference strain), resulting in correct identification in only 66.6% and 33.3%, respectively [25]. Masih et al. used the Bruker Biotyper database (OC version 3.1; updated January 2016) enhanced with an in-house fungal database to increase the number of identifiable *Aspergillus* species and reported successful MALDI-TOF MS identification results (score value of 2.0) for 97.7% of 45 *Aspergillus* isolates with clinical importance [30]. Normand et al. have used both in-house and Bruker reference databases as well as a panel of 422 isolates associated to 126 species (including 155 isolates of 34 different *Aspergillus* species). In general, the results of their study were achieved by applying a decisional algorithm in which only the highest score of four spots was taken into a report with a 1.7 score [39]. Testing the complete panel provided a correct identification of 87.41% (in-house database) and 35.15% (Bruker database) of isolates [39]. In order to assess the performance of the Bruker Microflex™ MALDI-TOF MS instrument (Billerica, MA, USA), Stein et al. attempted to identify clinical and

reference strains of molds, employing three libraries: the Bruker mold library, the National Institutes of Health (NIH) library, and the mass spectrometry identification (MSI) online library [122]. The aim of the study was to compare those results with conventional and molecular identification methods [122]. In this study, 50 strains of *Aspergillus* from 13 different species were evaluated by the aforementioned systems. Of these, four strains (two *A. niger*, one *A. sydowii*, and one *A. glaucus*) were not identified by MALDI-TOF MS [122]. MALDI-TOF MS was able to separate *Aspergillus flavus* and *A. oryzae* at the species level. This is remarkable as these species are difficult to discriminate by the use of β -tubulin sequence analysis and thus require the use of a labor-intensive DNA-based technique for their differentiation [16]. Zhou et al. evaluated VITEK mass spectrometry (MS) V3.0 for the identification of *A. fumigatus* using extracted fungal ribosomal proteins. Besides, they also examined the role of incubation time (48, 72, 96, and 120 h) and culture media (Sabouraud liquid media and Sabouraud and Chalet agar media). Of all strains, 88.5% (46/52) were discriminated as *A. fumigatus*, while the remaining 11.5% (6/52) results were inconsistent with that of morphological analysis. According to statistical analysis, the culture medium and the incubation temperature have no effects on the accuracy of identification process [123]. Vidal-Acuña et al. analyzed one hundred and ninety strains (179 clinical isolates and the 11 reference strains) blindly by the MALDI-TOF MS technology to appraise their generated in-house reference database. A 100% correlation was achieved with MALDI-TOF MS and sequencing methods [124]. Li et al. assessed the correctness of the Bruker Biotyper MALDI-TOF MS system for the identification of 381 *Aspergillus* clinical isolates representing 21 different *Aspergillus* species that were identified by molecular analysis. The Bruker Biotyper MALDI-TOF MS system was able to identify 30.2% (115/381) of the isolates to the species level and 49.3% to the genus level. By lowering the cutoff value from ≥ 2.000 to ≥ 1.700 , they improved the identification of the species level from 30.2 to 79.5% [125]. Nakamura et al. identified *Aspergillus* species by analyzing ribosomal subunit protein typing technique employing MALDI-TOF MS. They were able to identify the different species of *Aspergillus* as well as species within section Fumigati [126]. In summary, the reported *Aspergillus* identification rates by MALDI-TOF MS vary from 30.2 to 100% depending on the study.

Fusarium species

There has been an increase in the incidence of infections caused by *Fusarium* which belong to different species complex causing serious infections [32, 127]. More than 70 *Fusarium* species have also been documented as opportunistic human pathogens. *Fusarium* sp. cause both superficial

infections (e.g., keratitis and onychomycosis) and invasive and disseminated infections [32]. Triest et al. developed a MALDI-TOF MS identification approach relying upon an in-house database, which was constructed with spectra from 289 validated strains belonging to 40 *Fusarium* species [128]. Furthermore, they analyzed 19 species represented by more than one strain using MALDI Biotyper 3.0 software. Their results have shown that 82.8% and 91% were correctly identified at the species level by score value of 2 and 1.7, respectively [128]. Sleiman et al. evaluated 45 *Fusarium* isolates from six different species including *F. chlamydo-sporum* ($n=4$), *F. dimerum* ($n=3$), *F. fujikuroi* ($n=13$), *F. incarnatum-equiseti complex* ($n=4$), *F. oxysporum* ($n=9$), and *F. solani* ($n=12$) by Bruker library alone, and Bruker library plus in-house database incorporating in-house-built database with Bruker Filamentous Fungi Library has significantly improved the identification process with 84% accurate identification, compared to 42% success using Bruker library alone [32].

Scedosporium species

Scedosporium (*Pseudallescheria*) is a worldwide fungus that is most commonly found in contaminated water and soil. *Scedosporium* species, after *A. fumigatus*, are the most important colonized mold in the respiratory tract of cystic fibrosis patients [129, 130]. Molecular studies performed on *Scedosporium* species identified *S. prolificans* and *S. apiospermum* complex [131]. Since the degree of susceptibility to antifungal drugs varies in *Scedosporium* species, it is important to identify the species for appropriate treatment [132]. Recent studies have shown that the MALDI-TOF MS technique is very effective in detecting filamentous fungi such as *Scedosporium* [133, 134]. Outstandingly, *S. prolificans* was considered only distantly related to *Scedosporium* species, and it was reclassified as *Lomentospora prolificans*, and the genus *Lomentospora* reintroduced for this species [135]. Sitterlé et al. using 47 *Scedosporium* reference strains including *P. boydii* ($n=20$), *S. apiospermum* ($n=11$), *S. aurantiacum* ($n=4$), *P. minutispora* ($n=2$), *S. dehoogii* ($n=5$), and *L. prolificans* ($n=5$) prepared a suitable database for the detection of suspected clinical specimens of *Scedosporium* by MALDI-TOF MS (formic acid method) technique (Fig. 2) [136]. Then, to evaluate the validity of this database, using the MT-MS technique and gene sequence, 64 clinical and environmental samples were examined, and the results of each method were completely consistent [136]. In another study, which was performed on different clinical specimens using a pre-prepared database, *P. boydii* was identified from a patient sputum sample, which was confirmed by gene sequencing [134]. According to studies, the MALDI-TOF MS technique has high accuracy and sensitivity for the detection of *Scedosporium* species [134, 136].

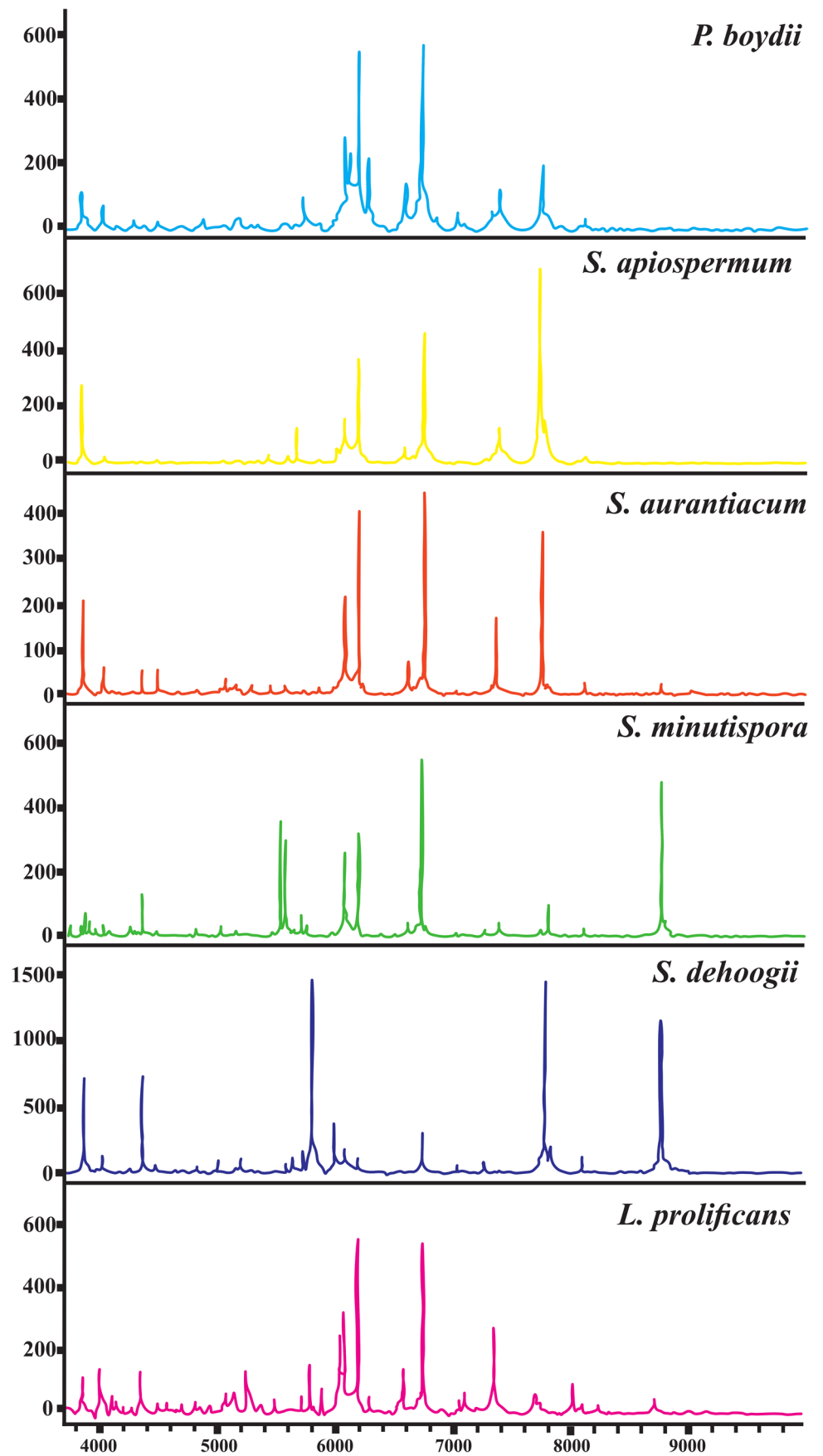
Mucorales species

Mucormycosis is the second most common invasive filamentous infection that occurs in patients who are immunocompromised or suffering from diabetes or trauma [134, 137]. Fungi of the order Mucorales, including *Rhizopus*, *Lichtheimia*, *Mucor*, *Syncephalastrum*, *Rhizomucor*, and *Cunninghamella*, are the causative agents of mucormycosis [138]. Shao et al. analyzed 111 isolates from six genera of Mucorales by MALDI-TOF MS in the Bruker Filamentous Fungi library v1.0 alone and in combination with BMU database. They, respectively, identified 55 (49.5%) and 90 (81.1%) isolates at the species level as well as 74 (66.7%) and 111 (100%) at genus levels [41]. De Carolis et al. evaluated MT-MS for species identification through direct surface analysis of the fungal strains including Mucorales. Eight isolates representing four different Mucorales genus (*Rhizopus*, *Lichtheimia*, *Mucor*, *Rhizomucor*) were fully identified [20]. Schrödl et al. have concentrated only on the genus *Lichtheimia*, which is one of the most common causative agents of mucormycosis. Forty-four strains of different *Lichtheimia* species (*L. corymbifera*, *L. ramosa*, *L. ornata*, *L. hyalospora*, and *L. sphaerocystis*) were used, including 34 clinical isolates [138]. Their result confirmed that the type of strains of all five species of *Lichtheimia* could be easily differentiated from other clinically important genus of Mucorales [138]. Dolatabadi et al. employed MALDI-TOF MS to differentiate between *Rhizopus microsporus* and *R. arrhizus* (*R. arrhizus* ($n=25$) and *R. microsporus* ($n=13$)). Discrimination of all *Rhizopus* isolates at species and variety levels was confirmed [139].

Dermatophyte species

The dermatophytes are able to invade keratinized tissue including the skin, hair, and nails, though they are usually limited to the nonliving cornified layer of the epidermis due to the presence of the immune system in immunocompetent hosts [56]. Recently, based on the new multilocus phylogenetic taxonomy, dermatophytes are categorized into seven genera: *Trichophyton*, *Microsporum*, *Epidermophyton*, *Lophophyton*, *Paraphyton*, *Nannizzia*, and *Arthroderma* [140]. Until now, several studies have investigated MALDI-TOF MS as a method for identifying dermatophytes. The reported accuracy rates of identification by MT-MS vary from 13.5 to 100% for dermatophytes [15, 54, 56–60, 141–146]. The effective identification of dermatophyte species depends on the number and variety of isolates available in the reference spectral library [53]. Owing to the substantial constraints of the commercial reference spectral libraries, most of the previous studies have introduced in-house reference libraries [51–53, 55, 56, 61, 62, 142]. Hence,

Fig. 2 MALDI-TOF mass spectra obtained from isolates of the *P. boydii*, *S. apiospermum*, *S. aurantiacum* sloe: *P. minutispora*, *S. dehoogii*, *L. prolificans*



there is a necessity to develop a commercial reference spectral library that comprises a broad spectrum of different strains. In several studies, the rate of correct identification has been reported above 90% for different species of dermatophytes [53, 61, 62, 142, 146]. In contrast, Karabicak et al. (13.5%) and Theel et al. (36.8%) obtained frustrating results by manufacturer reference spectra library [52, 60]. The results have been improved in the two studies mentioned above by using supplemented manufacturer reference spectra library and decreasing the log score from 2 to 1.7 [52, 60]. The use of MALDI-TOF MS to discriminate between the closely related species of the *T. mentagrophytes* complex was performed by Packeu et al. and Suh et al. [142, 146]. Their results indicated that the data obtained by MALDI-TOF MS had an acceptable correlation with phylogenetic data [142, 146]. Several factors involved in MALDI-TOF MS including culture medium, incubation time, protein extraction method, matrix type, mass spectrometer instrument, and reference spectra library [141]. For dermatophytes, MALDI-TOF MS has proven to be unaffected by the listed above variations except of reference spectra library [141]. The major restriction remains the insufficient representation of dermatophyte species in the reference spectra libraries of current commercialized MALDI-TOF MS identification systems. There is a necessity for expanding the manufacturer reference spectra library by employing an in-house reference library as a supplementary data, with the aim to encompass both inter- and intra-specific dermatophyte diversity. Nevertheless, this procedure requires skilled mycologists [141]. meanwhile, only few centers have the possibility to have access to this technology [141]. Hollemeyer et al. described a direct MALDI-TOF MS-based identification of dermatophytosis from clinical samples [147]. This method is high-speed and without requiring a cultivation step [147].

Black fungi

Melanized or black fungi are among etiologic agents of cutaneous to systemic diseases with high mortality. Furthermore, infections caused by black fungi are commonly underestimated, and there is inadequate clinical experience with these fungi. About 130 species from 70 genera have been documented to be related to infections in humans and animals [148]. Singh et al. examined 21 genera consisted of 29 species, predominantly within the orders *Pleosporales* and *Chaetothyriales*. Among the 29 fungal species, only 6 (20%) species were identified by the MALDI-TOF MS Bruker Biotyper, version 3.1 commercial database of Bruker Daltonics. However, a 100% identification rate of 20 additional species was performed in this study by establishing an in-house database [35]. Özhak-Baysan et al. analyzed the applicability of MT-MS for the identification of *Exophiala* species. The investigation consisted of a total of 110 *Exophiala* isolates, involving 15 CBS strains representing 4 species, *Exophiala dermatitidis* ($n=61$), *E. phaeomuriformis* ($n=36$), *E. crusticola* ($n=9$), and *E. heteromorpha* ($n=4$). All *Exophiala* strains were identified to the genus and species levels, with identification rates of 96.8% and 90.5%, respectively [149]. Kondori et al. evaluated MALDI-TOF MS for the identification of 51 *Exophiala dermatitidis* isolated from a patient with cystic fibrosis. The result showed more than 70% correct identification rates of all isolates by MALDI-TOF MS Saramis software package [28].

MALDI-TOF MS in antifungal resistance assay

Due to the spread of resistance to antifungal drugs among opportunistic fungi such as *Aspergillus* and *Candida*, accurate identification of the pathogen and timely initiation of appropriate treatment have a very important role in patient survival. Recently, the emergence of antifungal resistance strains has increased the importance of performing

Table 2 Overview of MALDI-TOF mass spectrometry for examining the presence or absence of drug resistance

Fungi	Antifungal agent	Principle of the method	Major errors (%)	References
<i>C. albicans</i>	Caspofungin, fluconazole, voriconazole, posaconazole	Exposure of the tested fungus in different dilutions of specific antifungal agents for several hours.	17.8	[153, 155]
<i>C. glabrata</i>	Caspofungin, fluconazole, voriconazole, posaconazole	Mass spectrum profile change evaluation (MT-MS method)	2.12	[151, 155]
<i>C. tropicalis</i>	Caspofungin, fluconazole, voriconazole, posaconazole		13.51	[68]
<i>C. krusei</i>	Caspofungin		0	[151]
<i>A. fumigatus</i>	Caspofungin		0	[151]
<i>A. flavus</i>	Caspofungin		0	[155]
<i>C. parapsilosis</i>	Caspofungin		0	[151]
<i>C. glabrata</i>	Caspofungin	MBT ASTRA	5.09	[68, 151]
<i>C. albicans</i>	Caspofungin	MBT ASTRA	12.28	[151]

Table 3 Determination and characterization of mycotoxins by MALDI-TOF mass spectrometry

Mycotoxin	Molecular weight	The peak value in <i>F. verticillioides</i>	The peak value in <i>F. proliferatum</i>	The peak value in <i>F. oxysporum</i>
Fumonisin B1	722.4	35,000	42,000	38,000
Fumonisin B2	706.4	10,000	11,000	55,000
Zearalenone	319	170,000	90,000	140,000
Nivalenol	313.1	90,000	2300	260,000
3-Acetyl-deoxynivalenol	339	56,000	24,000	13,000
Aflatoxin G1	329	6800	37,000	50,000

the antifungal susceptibility test (AFST) on isolates from infected patients in clinical practices [150]. Cultural and microscopically identification of the pathogen and then the examination of growth or non-growth in the presence of the antifungal drug to evaluate drug susceptibility require more than 48 h [150]. Although the introduction of molecular methods into clinical laboratories has significantly reduced the time of microorganism's identification and drug resistance evaluation, these methods are expensive and culture-dependent and require experts in this field. The MALDI-TOF MS method is one of the latest achievements in the molecular diagnostic method for detecting the etiological agents and examining the presence or absence of drug resistance, which is very fast, economical, and simple (Table 2) [68, 151–155].

Several studies using the MALDI-TOF MS technique have provided alternative methods to the CLSI and EUCAST AFST reference methods. One method of assessing drug susceptibility in fungi by MALDI-TOF MS is to expose the fungus to serial dilution of certain antifungals (such as fluconazole and caspofungin) for 15 h. At a certain concentration of antifungal, there is a significant change in the mass spectra profile. The lowest antifungal concentration at which the spectra change is called the minimum profile change concentration (MPCC), which is equivalent to the MIC obtained by the CLSI method [151, 154, 155]. In Marinach et al. study, there were no differences between MPCC and MIC

value [150]. However, by this method, MPCC was obtained for other fungi including other *Candida* species against caspofungin that MPCC was 100% MIC-compliant [153]. In the study by Vatanshenassan et al., the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT ASTRA) method, previously used to detect antibacterial resistance, was used to identify caspofungin resistance in yeasts such as *C. albicans* and *C. glabrata* [151]. It takes about 5–6 h to determine the status of drug susceptibility. The results of the drug susceptibility test by MBT ASTRA method were 100% consistent with the MIC results by CLSI method. Up to now, the MALDI-TOF MS technique has been used to identify drug resistance in various fungi, including *Aspergillus* and *Candida* species (Table 2). Although the MALDI-TOF MS technique is fast and useful for detecting drug resistance, it has an error in detecting some resistant species compared to the MIC obtained by CLSI [153, 155].

MALDI-TOF MS in mycotoxin detection

Fungi produce many secondary metabolites, and one of the most important metabolites is mycotoxins such as fumonisins, trichothecenes, zearalenone, aflatoxins, and ochratoxins. Mycotoxins may accumulate in contaminated crops and cause a disease called mycotoxicosis in humans and animals after ingestion. As fungi, molds, and yeast, which secrete mycotoxins, are ubiquitous, mycotoxins

Table 4 Using MALDI-TOF in mycobiome research

No	Author, year	Country	Mycobiome	No. of isolates	Correct identification	MALDI-TOF system	Organism
1	Samb-Ba et al. 2014 [164]	Senegal	Gut mycobiome	3	100%	MALDI Biotyper	<i>C. albicans</i>
2	Aslani et al. 2018 [80]	Iran	Oral mycobiome	162	100%	MALDI Biotyper 3.0	Different yeast species
3	Starý et al. 2020 [165]	Czech Republic	Gut mycobiome	16	100%	MALDI Biotyper	<i>C. albicans</i>
4	Griessler et al. 2021 [166]	Germany	Oral mycobiome	1	100%	MALDI-TOF	<i>C. albicans</i>
5	Lu et al. 2021 [167]	Taiwan	Commensal oral yeasts	320	96.9%	MALDI Biotyper 3.0	Different yeast species

are among the most important contaminants in foods. Mycotoxins have become a major issue for essential food products over the past two decades. The wide range and frequent occurrence of mycotoxins naturally in crops demonstrated the importance of studying the mycotoxigenic fungi [156–159].

Detecting the presence of mycotoxins requires a sensitive, uncomplicated, and accelerated assay. Several highly sensitive and accurate analytical methods have been used to analyze mycotoxins, for instance, high-performance liquid chromatography (HPLC). Liquid chromatography-mass spectrometry (LC-MS) and MALDI-TOF methods are helpful for determining mycotoxins with quantitative outcomes (Table 3) [160, 161]. MALDI-TOF MS is a highly sensitive method for analysis that can be used to evaluate low concentrations of peptides. So far, this technique has been used to identify mycotoxins produced by *Fusarium* species, which is one of the main contaminants of agricultural products. *F. verticillioides*, *F. proliferatum*, and *F. oxysporum* have been studied for mycotoxins fumonisin, aflatoxin, 3-acetyl-deoxynivalenol, nivalenol, and zearalenone [160, 161].

MALDI-TOF MS in mycobiome research

The human mycobiome, referring mainly to all the fungal biota living inside the human body sites, is an important component of the human microbiome. These diverse biota interact with other commensal microbiomes, as well as with the host [162]. Changes in the population of these mycobiomes are probably contributing to the progression of various diseases such as inflammatory bowel disease (IBD) and play a very important role in disease or health in humans [163].

Despite its importance, few studies have been done in this area. Recently, new approaches developed to determine mycobiomes diversity, such as next-generation sequencing methods and MALDI-TOF MS, are greatly broadening our view of fungal importance.

MALDI-TOF MS analyses being fast, easy, inexpensive, and accurate and the fact that it can be applied to detect a comprehensive array of mycobiome imply that the method would be very suitable for future workers studying the mycobiome. Table 4 presents some studies that perform on human mycobiomes [80, 164–167].

Conclusion

MALDI-TOF MS has developed as a routine method for the identification of yeasts and filamentous fungi and is also applicable to the detection of mycotoxins and antifungal drug resistance of pathogenic fungi, although in order to

include reference spectra of unusual pathogens, always up-to-date databases are indispensable. Since fast and accurate identification of fungal pathogens is significantly beneficial for optimal patient treatment, MALDI-TOF MS-based identification should be integrated into antifungal stewardship strategies. The implementation of MALDI-TOF MS for intra-specific typing holds great promise, as it has shown the ability to detect mycotoxins, drug-resistant phenotypes, mycobiome research, biofilm-producing strains, and potentially cluster isolates for epidemiological purposes.

Declarations

Conflict of interest The authors declare no competing interests.

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