Food Microbiology 81 (2019) 76–88

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Development and application of MALDI-TOF MS for identification of food spoilage fungi

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ARTICLE INFO

Article history:
Received 4 December 2017
Received in revised form
30 March 2018
Accepted 1 May 2018
Available online 2 May 2018

Keywords:
MALDI-TOF MS
Identification
Filamentous fungi
Food spoilage

ABSTRACT

Filamentous fungi are frequently involved in food spoilage and cause important food losses and substantial economic damage. Their rapid and accurate identification is a key step to better manage food safety and quality. In recent years, MALDI-TOF MS has emerged as a powerful tool to identify microorganisms and has successfully been applied to the identification of filamentous fungi especially in the clinical context. The aim of this study was to implement a spectral database representative of food spoilage molds. To this end, after application of a standardized extraction protocol, 6477 spectra were acquired from 618 fungal strains belonging to 136 species and integrated in the VITEK MS database. The performances of this database were then evaluated by cross-validation and ~95% of correct identification to the species level was achieved, independently of the cultivation medium and incubation time. The database was also challenged with external isolates belonging to 52 species claimed in the database and 90% were correctly identified to the species level. To our best knowledge, this is the most comprehensive database of food-relevant filamentous fungi developed to date. This study demonstrates that MALDI-TOF MS could be an alternative to conventional techniques for the rapid and reliable identification of spoilage fungi in food and industrial environments.

1. Introduction

Fungi are ecologically, physiologically and morphologically diverse and constitute one of the largest group of organisms on earth with at least 1.5 million and more probably, close to 3 million species (Hawksworth, 2015). Among fungi, microscopic filamentous fungi (also called molds) have an important impact on human activities, either positive or negative. On one hand, they are important producers of enzymes, organic acids and antibiotics and can be used to manufacture fermented foods (Chalupová et al., 2014). On the other hand, they are able to spoil a large variety of food and feed commodities, from raw materials to finished products. Their growth can lead to several types of product-alteration such as taste and odor problems and decay. *Penicillium* and *Aspergillus* spp. are the most common spoilage fungi, whereas *Fusarium* species are mostly responsible for significant yield losses of small grain cereals and maize (Pitt and Hocking, 2009). Certain members of these genera as well as species from other genera can also potentially produce mycotoxins (Waśkiewicz, 2014), some of which have adverse effects on human and animal health and can thus render food and feed improper for consumption. Regarding their impact on human activities, it has been estimated that 5–10% of the worldwide food losses is due to fungal spoilage (Filtenborg et al., 1996). In this context, correct identification of food spoilage fungi

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https://doi.org/10.1016/j.fm.2018.05.001
0740-0020/

is a key step to better manage food safety and quality. An erroneous identification could lead to risk under-estimation or unnecessary counter measures (Hawksworth, 2015; Schnürer and Magnusson, 2005). It is also worth mentioning that many spoilage filamentous fungi are also opportunistic human pathogens and their identification at the species-level could also be of interest in the clinical context.

Identification of fungal isolates is based on phenotypic and/or genotypic methods. Phenotypic identification generally involves the examination of different morphological features at both macroscopic and microscopic levels, such as the thallus aspect or that of asexual spore-bearing structures, after a cultivation step on agar medium. Biochemical characteristics such as secondary metabolite production can also be used as a complementary approach (Houbraken et al., 2007). Genotypic methods based on molecular biology are considered the gold-standard for mold identification because of their high specificity. Indeed, comparative sequence analysis of conserved genes but also of unique DNA regions allows establishing the identity and phylogeny among organisms. For example, the internal transcribed spacer (ITS) region in ribosomal operons is considered as the primary barcode for fungi (Schoch et al., 2012). However, in some groups, other genes are superior to ITS for species-level identification such as the β-tubulin gene for *Penicillium* spp. (Visagie et al., 2014) and elongation-factor gene for *Fusarium* spp. (Geiser et al., 2004). Moreover, single-locus approaches are not always sufficient for accurate identification at the species-level. Although DNA sequencing allows good identification, it can be costly, fastidious and it always requires expertise (Chalupová et al., 2014). Thus, there is a continuous need for more rapid techniques to identify filamentous fungi, which are as reliable as molecular tools.

In recent years, Matrix-Assisted Laser Desorption Ionisation – Time Of Flight Mass Spectrometry (MALDI-TOF MS) has emerged as an alternative for or supplement to conventional phenotypic and molecular identification tools. Since then, the efficacy of MALDI-TOF MS for bacterial species identification has been demonstrated in an exponentially growing number of publications, mainly focusing on clinically relevant species (Basile et al., 1998; Fournier et al., 2012; Nacef et al., 2017; Nagy et al., 2009; van Baar, 2000; van Wuijckhuijse et al., 2005).

Over the last fifteen years, MALDI-TOF MS has also been successfully applied to filamentous fungi identification. The main difference is that unlike most bacteria, fungal mycelium, fructification organs and spores can have a thick cell wall thus an extraction step is often required before spectra acquisition (Cassagne et al., 2011). After Welham et al. (2000) who applied MALDI-TOF MS for fungal identification for the first time, several studies showed that this method could be adapted for the identification of various fungal groups including *Penicillium*, *Aspergillus* and *Fusarium* spp. For instance, Chen and Chen (2005) analyzed intact spores of *Penicillium* spp. while Hettick et al. (2008) could successfully identify 12 *Penicillium* spp. with an extraction step before analysis. Marinach-Patrice et al. (2009) were able to correctly identify 92% of *Fusarium* spp. belonging to 9 different species, and Rodrigues et al. (2011) applied MALDI-TOF MS on species from the *Aspergillus* section *Flavi*. Finally, a recent study of Rychert et al. (2017) demonstrated the accuracy of MALDI-TOF MS by successfully identifying 91% of ~1400 clinical fungal isolates. Even though these results were promising, they were obtained using different protocols (either using intact spores or after an extraction step). In addition, except for the study of Rychert et al. (2017) which was devoted to clinically relevant fungal species, all studies cited above focused on a single genus and/or a limited number of species.

Many studies and reviews have dealt with MALDI-TOF MS microbial identification in clinical contexts (Ge et al., 2016; van Belkum et al., 2015, 2017) and today, this technique is routinely used for bacterial identification. Despite the increasing number of publications dealing with spectral database implementation for clinically relevant fungal species (Gautier et al., 2014; Lau et al., 2013; Normand et al., 2017), a real effort is still needed to construct robust database for routine identification of food borne filamentous fungi with the use of standardized protocols (Sanguinetti and Posteraro, 2017). Therefore, the aim of this work was to implement a database for food spoilage fungi identification with 618 strains representing 136 species using a standardized extraction protocol.

2. Material and methods

2.1. Fungal strains and cultivation

2.1.1. Fungal strains used for database construction

A total of 618 strains corresponding to 34 genera and 136 species (Table 1), representing major mold species encountered in food and feed, were obtained from the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) and the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). These species were selected based on scientific watch (Chen et al., 2017; Garnier et al., 2017; Perrone et al., 2007; Pitt and Hocking, 2009; Santos et al., 2016) and three main criteria e.g., frequency of occurrence in food, number of spoiled food types and ability to produce mycotoxins. All strains were identified at the species-level by morphological analysis based on macroscopic and microscopic features. In addition, when possible, at least one strain per species included in the database had also been identified by DNA sequencing with 479 out of 618 strains identity confirmed by sequencing of one or more regions (ITS region, partial β-tubulin gene, partial translation elongation factor-1 alpha gene, partial *mcm7* and *tsr1* genes).

All strains were sub-cultivated on appropriate solid culture media to assess viability and purity before they were used for spectrum acquisition. Then, they were cultivated at 25 °C for 2 and 8 days on four different media: Sabouraud Dextrose Agar (bioMérieux, Marcy l'Etoile, France), Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Yeast Glucose Chloramphenicol agar (YGC). For the last three media, three suppliers were used, e.g., bioMérieux (Marcy l'Etoile, France), Becton Dickinson (Le Pont de Claix, France) and Oxoid (Dardilly, France). Different media and suppliers were chosen in order to represent the diversity of media used in industrial mycology and generate a database as robust as possible.

2.1.2. Fungal strains used for external database validation

For external database validation, 73 well characterized strains obtained from the "Laboratoire Universitaire de Biodiversité et Ecologie Microbienne" or UBOCC were used. These strains belonged to 67 different species (Table 2). Fifty-two species were represented in the database while 15 species were absent from it. None of the strains used for external validation were used to build the database. Each strain was cultivated under two different conditions. For each condition, an incubation time varying between 2 and 8 days and a cultivation medium, among all media and suppliers cited above, was randomly chosen.

2.2. Sample preparation

After cultivation, isolates were processed following the manufacturer's instructions. Hyphae and/or conidia were collected from the surface of agar plates (approximately 1 cm in diameter) with a moisturized sterile cotton swab (bioMérieux, Marcy l'Etoile, France). The biomass was then transferred into 900 µL of 70%

Table 1
Selected species and strain numbers for each species used to build the database.

Species	Strain number*
Actinomucor elegans	CBS 111556, UBOCC-A-101334, UBOCC-A-106035, UBOCC-A-101333
Alternaria alternata	CBS 916.96, CBS 117587, CBS 116329, CBS 115152, CBS 117143
Apergillus arachidicola	CBS 117610, CBS 117612, CBS 117611, CBS 117614, CBS 117615
Aspergillus calidoustus	UBOCC-A-101086, CBS 113228, CBS 112452, CBS 114380, CBS 121601
Aspergillus candidus	CBS 566.65, CBS 114385
Aspergillus carbonarius	UBOCC-A-105002, UBOCC-A-105005, UBOCC-A-105008, CBS 113.80, CBS 111.26
Aspergillus chevalieri	UBOCC-A-112066, UBOCC-A-112181, CBS 129.54, CBS 522.65, CBS 121704
Aspergillus fenneliae	CBS 584.90
Aspergillus flavus	UBOCC-A-106028, UBOCC-A-106032, UBOCC-A-106029, UBOCC-A-108068, UBOCC-A-106031, UBOCC-A-101061, UBOCC-A-106030, UBOCC-A-106033, UBOCC-A-101063, CBS 100927
Aspergillus foetidus	CBS 114.49, CBS 139.48, CBS 119383, CBS 121.28, CBS 122587
Aspergillus fumigatus	UBOCC-A-106001, UBOCC-A-114029, UBOCC-101066, UBOCC-A-106018, UBOCC-A-110089
Aspergillus glaucus	UBOCC-A-108077, CBS 117337, CBS 150.92
Aspergillus intermedius	CBS 117329, CBS 523.65, CBS 108.55, CBS 116.62, CBS 117315
Aspergillus lacticoffeatus	CBS 101884, CBS 101885, CBS 101886
Apergillus misclerotigenes	CBS 117635, CBS 117633, CBS 117634, CBS 117620, CBS 117639
Aspergillus montevidensis	CBS 117323, CBS 817.96, CBS 119376, CBS 289.95, CBS 518.65
Aspergillus nidulans	UBOCC-A-110152, UBOCC-A-101069, CBS 126972
Aspergillus niger	UBOCC-A-112064, UBOCC-101073, UBOCC-112080, UBOCC-A-112082, UBOCC-A-101075, UBOCC-A-101076, UBOCC-A-101072, UBOCC-112268, UBOCC-A-101074, CBS 554.65
Aspergillus novoparasiticus	CBS 126849, CBS 126850
Aspergillus ochraceus	UBOCC-A-111102, UBOCC-A-105011, CBS 547.65, CBS 588.68, CBS 108.08
Aspergillus parasiticus	UBOCC-A-111039, UBOCC-A-111041, UBOCC-A-111038, CBS 100926, CBS 100939
Aspergillus penicilloides	CBS 540.65
Aspergillus proliferans	UBOCC-A-101081, CBS 121.45, CBS 115.46
Aspergillus pseudoglaucus	UBOCC-A-112083, UBOCC-A-112117, UBOCC-A-112075, CBS 123574, CBS 117314
Aspergillus restrictus	CBS 541.65
Aspergillus ruber	CBS 135680, CBS 530.65, CBS 137.61, CBS 104.18
Aspergillus sydowii	UBOCC-A-108052, UBOCC-A-108050, UBOCC-A-110137, UBOCC-A-108053
Aspergillus tamarii	UBOCC-A-111046, UBOCC-A-111043, UBOCC-A-111045, CBS 129.49, CBS 104.14, CBS 104.13, CBS 590.68
Aspergillus terreus	UBOCC-A-101084, UBOCC-A-114003, CBS 116878, CBS 601.65, CBS 116757
Aspergillus thermomutatus	UBOCC-A-101204, CBS 208.92, CBS 100504, CBS 110899, CBS 117074
Aspergillus tubingensis	CBS 116417, CBS 115.29, CBS 626.66, CBS 134.48, CBS 117765
Aspergillus versicolor	UBOCC-A-111047, UBOCC-101087, UBOCC-102012, UBOCC-A-101088
Aspergillus wentii	UBOCC-A-111049, UBOCC-A-111051, UBOCC-A-101090, CBS 229.67, CBS 127.28
Aspergillus westerdijkiae	UBOCC-A-101078, UBOCC-A-105012, UBOCC-A-105006, CBS 121983, CBS 112803
Aureobasidium pullulans	UBOCC-A-101092, UBOCC-A-108056, UBOCC-A-108057, UBOCC-A-101091
Botrytis cinerea	UBOCC-A-101098, UBOCC-A-101099, CBS 810.69
Chaeotomium globosum	UBOCC-A-101012, CBS 148.60, CBS 148.51, CBS 107.14
Cladosporium cladosporioides	UBOCC-A-112148, UBOCC-A-101114, UBOCC-A-101109, UBOCC-A-111114, UBOCC-A-111115, UBOCC-A-101108
Cladosporium dominicanum	UBOCC-A-113071, UBOCC-A-113069, UBOCC-A-113070, CBS 119145
Cladosporium fusiforme	UBOCC-A-113072, CBS 119414
Cladosporium halotolerans	UBOCC-A-113073, UBOCC-A-113075, UBOCC-A-113074, CBS 139586, CBS 119416
Cladosporium herbarum	UBOCC-A-108074, UBOCC-A-101112, CBS 673.69
Cladosporium langeronii	UBOCC-A-112121, UBOCC-A-112132, UBOCC-A-112084, CBS 123171, CBS 601.84
Cladosporium oxysporum	CBS 125991, CBS 120813
Cladosporium psychrotolerans	UBOCC-A-113077, UBOCC-A-113078, CBS 119412
Cladosporium ramotenellum	UBOCC-A-108073, UBOCC-A-108072, CBS 109501, CBS 118.24, CBS 109031
Cladosporium sphaerospermum	UBOCC-A-112116, UBOCC-A-108054, UBOCC-A-101110, UBOCC-A-101111
Cladosporium velox	UBOCC-A-113082, UBOCC-A-113081, CBS 119417
Didymella glomerata	UBOCC-A-109091, CBS 834.84, CBS 133.72, CBS 304.49, CBS 134109
Didymella pinodella	CBS 318.90, CBS 531.66, CBS 133.92, CBS 123522, CBS 403.65
Epicoccum nigrum	UBOCC-A-113084, UBOCC-A-101131
Fusarium acuminatum	UBOCC-A-109036, UBOCC-A-109086, CBS 102796, CBS 140913, CBS 680.74
Fusarium arthrosporioides	UBOCC-A-109001, UBOCC-A-109049, UBOCC-A-109034
Fusarium avenaceum	UBOCC-A-109018, UBOCC-A-101136, UBOCC-A-109048, UBOCC-A-109141, UBOCC-A-109012, CBS 408.86
Fusarium cerealis	CBS 195.80, CBS 832.85, CBS 589.93
Fusarium culmorum	UBOCC-A-107001, UBOCC-A-109110, UBOCC-A-109108, UBOCC-A-109123, UBOCC-A-101139
Fusarium domesticum	UBOCC-A-109095, UBOCC-A-113010, CBS 244.82
Fusarium equiseti	UBOCC-A-109041, UBOCC-A-109029, UBOCC-A-109045, UBOCC-A-109030, UBOCC-A-109097, UBOCC-A-109016
Fusarium graminearum	UBOCC-A-109032, UBOCC-A-109011, UBOCC-A-109106, UBOCC-A-109026, UBOCC-A-109130, UBOCC-A-101143
Fusarium incarnatum	UBOCC-A-111066, CBS 163.57, CBS 161.25, CBS 791.70
Fusarium langsethiae	UBOCC-A-110061, UBOCC-A-110063, UBOCC-A-110062, UBOCC-A-109148, CBS 113234
Fusarium lateritium	UBOCC-A-102014, UBOCC-A-101146, UBOCC-A-101147
Fusarium oxysporum	UBOCC-A-108128, UBOCC-A-109131, UBOCC-A-101157, UBOCC-A-109009, CBS 221.49
Fusarium poae	UBOCC-A-109111, UBOCC-A-109135, UBOCC-A-109113, UBOCC-A-109136, UBOCC-A-109021

Table 1 (*continued*)

Species	Strain number*
Fusarium proliferatum	UBOCC-A-109147, UBOCC-A-109134, UBOCC-A-109028, UBOCC-A-109133
Fusarium sambucinum	UBOCC-A-109024, UBOCC-A-109020, UBOCC-A-109027, UBOCC-A-109019
Fusarium solani	UBOCC-A-109146, UBOCC-A-110136, UBOCC-A-114086, CBS 181.29
Fusarium sporotrichioides	UBOCC-A-109116, UBOCC-A-109114, UBOCC-A-109132, UBOCC-A-109115, UBOCC-A-102015
Fusarium subglutinans	UBOCC-A-111070, UBOCC-A-109087, CBS 119831, CBS 747.97, CBS 215.76
Fusarium temperatum	UBOCC-A-109150, UBOCC-A-109151, UBOCC-A-101148, CBS 138287
Fusarium tricinctum	CBS 119842, CBS 253.50, CBS 119841, CBS 393.93, CBS 410.86
Fusarium venenatum	UBOCC-A-109013, UBOCC-A-109003, CBS 148.95, CBS 140949, CBS 127.95
Fusarium verticillioides	UBOCC-A-109145, UBOCC-A-109122, UBOCC-A-109118, UBOCC-A-109121, CBS 218.76, CBS 447.95
Geotrichum candidum	UBOCC-A-101172, UBOCC-A-101173, UBOCC-A-108081, UBOCC-A-101169, CBS 615.84
Lasiodiplodia theobromae	CBS 110495, CBS 112874, CBS 124.13, CBS 118843
Lichtheimia corymbifera	UBOCC-A-102023, UBOCC-A-101328
Macrophomina phaseolina	CBS 460.70, CBS 121.82, CBS 162.25, CBS 227.33, CBS 416.62
Microdochium majus	CBS 121295
Microdochium nivale	UBOCC-A-105026, UBOCC-A-105027
Mucor circinelloides	UBOCC-A-109188, UBOCC-A-109183, UBOCC-A-109192, UBOCC-A-109182
Mucor fragilis	UBOCC-A-109071, UBOCC-A-101356, UBOCC-A-109199
Mucor hiemalis	UBOCC-A-101360, UBOCC-A-101359, UBOCC-A-109059
Mucor lanceolatus	UBOCC-A-109074, UBOCC-A-101355, UBOCC-A-109158, UBOCC-A-109153, UBOCC-A-101148, UBOCC-A-101329
Mucor plumbeus	UBOCC-A-102004, UBOCC-A-103032, UBOCC-A-109053, UBOCC-A-108086, UBOCC-A-109152, UBOCC-A-109061, UBOCC-A-101363, UBOCC-A-109052, CBS 129.41
Mucor racemosus	UBOCC-A-109155, UBOCC-A-109186, UBOCC-A-109162, UBOCC-A-109213, UBOCC-A-109212, UBOCC-A-109159, UBOCC-A-101352, UBOCC-A-109177, CBS 113.08, CBS 260.68
Paecilomyces divaricatus	CBS 113956, CBS 131275, CBS 110429, CBS 113955
Paecilomyces formosus	CBS 990.73B, CBS 628.66, CBS 296.93, CBS 113248, CBS 121584
Paecilomyces fulvus	UBOCC-A-101005, CBS 135.62, CBS 113245, CBS 146.48, CBS 132.33
Paecilomyces saturatus	UBOCC-A-101210, CBS 251.55, CBS 492.84, CBS 990.73A
Paecilomyces variotii	UBOCC-A-106027, UBOCC-A-112156, UBOCC-A-103044, UBOCC-A-110151, CBS 101032
Penicillium adametzioides	CBS 140499, CBS 313.59
Penicillium antarcticum	CBS 100492, CBS 116938, CBS 121927, CBS 100491, CBS 116939
Penicillium aurantiogriseum	UBOCC-A-110001, UBOCC-A-101384, UBOCC-A-111185, UBOCC-A-111184, UBOCC-A-101385
Penicillium bialowiezense	CBS 227.28, CBS 116044, CBS 110104, CBS 110102, CBS 118865
Penicillium brevicompactum	UBOCC-A-108094, UBOCC-A-110065, UBOCC-A-101389, UBOCC-A-110008, UBOCC-A-108095, UBOCC-A-110007, UBOCC-A-108093, UBOCC-A-101383, UBOCC-A-111022
Penicillium camemberti	UBOCC-A-102018, UBOCC-A-112058, UBOCC-A-113026, UBOCC-A-113011, UBOCC-A-108097, UBOCC-A-101455, UBOCC-A-101392, UBOCC-A-113060, UBOCC-A-108125, CBS 299.48
Penicillium carneum	UBOCC-A-111171, CBS 112489, CBS 468.95, CBS 466.95
Penicillium chermesinum	UBOCC-A-111054, UBOCC-A-112184, UBOCC-A-112188
Penicillium chrysogenum	UBOCC-A-110067, UBOCC-A-114030, UBOCC-A-106024, CBS 111214, CBS 478.84
Penicillium citreonigrum	UBOCC-A-113039, UBOCC-A-111242, UBOCC-A-112169, UBOCC-A-110143
Penicillium citrinum	UBOCC-A-111025, UBOCC-A-111010, UBOCC-A-111159, CBS 309.48, CBS 252.55
Penicillium commune	UBOCC-A-108098, UBOCC-A-111014, CBS 311.48, CBS 115505, CBS 343.51
Penicillium corylophilum	UBOCC-A-109224, UBOCC-A-109219, UBOCC-A-112193, UBOCC-A-109222, UBOCC-A-101405
Penicillium crustosum	UBOCC-A-101407, UBOCC-A-110068, UBOCC-A-112189, UBOCC-A-111011, UBOCC-A-108101, UBOCC-A-108100, CBS 115503
Penicillium decumbens	UBOCC-A-112118, UBOCC-A-112067, UBOCC-A-113037, UBOCC-A-112072, UBOCC-A-111240
Penicillium dierckxii	UBOCC-A-111013, CBS 304.48, CBS 172.44, CBS 229.81
Penicillium digitatum	UBOCC-A-111019, UBOCC-A-111064, UBOCC-A-111032, UBOCC-A-111015, UBOCC-A-101408
Penicillium discolor	CBS 474.84, CBS 969.97, CBS 551.95, CBS 547.95, CBS 112568
Penicillium expansum	UBOCC-A-101452, UBOCC-A-101410, UBOCC-A-110032, UBOCC-A-110028, UBOCC-A-110023, UBOCC-A-110030, UBOCC-A-110034, UBOCC-A-101406, UBOCC-A-110024, CBS 325.48
Penicillium fuscoglaucum	UBOCC-A-108104, UBOCC-A-108129, UBOCC-A-108127
Penicillium glabrum	UBOCC-A-108114, UBOCC-A-108105, UBOCC-A-109089, UBOCC-A-109098, UBOCC-A-108107
Penicillium italicum	UBOCC-A-112151, UBOCC-A-110043, UBOCC-A-111228, UBOCC-A-101426, UBOCC-A-110044
Penicillium nalgiovense	UBOCC-A-112103, UBOCC-A-112101, UBOCC-A-113013, UBOCC-A-101430, UBOCC-A-101431
Penicillium nordicum	UBOCC-A-112105, UBOCC-A-112106, CBS 483.84, CBS 110770, CBS 323.92
Penicillium oxalicum	UBOCC-A-101438, UBOCC-A-102021, UBOCC-A-101437, UBOCC-A-111029, UBOCC-A-101436, UBOCC-A-101435
Penicillium palitans	UBOCC-A-112147, UBOCC-A-113024, UBOCC-A-113023, UBOCC-A-101387, CBS 311.48
Penicillium paneum	UBOCC-A-101450, UBOCC-A-109218, UBOCC-A-111183, UBOCC-A-101448, CBS 303.97
Penicillium polonicum	UBOCC-A-102002, UBOCC-A-101429, CBS 116689, CBS 222.28, CBS 112650
Penicillium roqueforti	UBOCC-A-111170, UBOCC-A-111188, UBOCC-A-111192, UBOCC-A-111191, UBOCC-A-111211, UBOCC-A-111033, UBOCC-A-111194, UBOCC-A-109090, UBOCC-A-111187, CBS 221.30
Penicillium salamii	CBS 135391, CBS 135392, CBS 135395, CBS 135393, CBS 135396
Penicillium simplicissimum	UBOCC-A-101427, UBOCC-A-111189, UBOCC-A-101428
Penicillium solitum	UBOCC-A-113015, UBOCC-A-108113, UBOCC-A-113038, UBOCC-A-111055, CBS 141.86
Penicillium verrucosum	UBOCC-A-105007, UBOCC-A-109221, UBOCC-A-105014, UBOCC-A-105004, CBS 115508
Penicillium viridicatum	UBOCC-A-111198, UBOCC-A-111200, UBOCC-A-111199, UBOCC-A-111201, UBOCC-A-108115
Purpureocillium lilacinum	UBOCC-A-108030, UBOCC-A-108014, UBOCC-A-108027, UBOCC-A-111264, UBOCC-A-101208
Rhizopus oryzae	UBOCC-A-101372, CBS 146.90, CBS 278.38, 515.94, CBS 127.08
Rhizopus stolonifer	UBOCC-A-108116, CBS 382.52, CBS 819.96, CBS 109.76
Scopulariopsis fusca	UBOCC-A-108120, UBOCC-A-101272, UBOCC-A-108119, UBOCC-A-101271, UBOCC-A-113016
Talaromyces bacillisporus	UBOCC-A-101035, CBS 296.48, CBS 116927, CBS 110774, CBS 158.67
Talaromyces macrosporus	CBS 317.63, CBS 118884, CBS 130.89

(*continued on next page*)

Table 1 (*continued*)

Species	Strain number*
Trichoderma harzianum	UBOCC-A-112175, CBS 226.95
Trichoderma viride	UBOCC-A-111251, UBOCC-A-111252, UBOCC-A-101292
Trichothecium roseum	UBOCC-A-101293, CBS 281.28, CBS 566.50
Umbellopsis isabellina	CBS 208.32, CBS 100559, CBS 560.63
Wallemia sebi	UBOCC-A-110069, UBOCC-A-112060, UBOCC-A-101324, CBS 818.96, CBS 184.28

*UBOCC, Université de Bretagne Occidentale Culture Collection; CBS, Centraalbrueau voor Schimmelcultures Collection; Underlined strain number indicates that strain identification was confirmed by DNA-sequencing.

ethanol (Carlo Erba, Val de Reuil, France) in a microcentrifuge tube, vortexed for 5 s and then centrifuged at 14,000 × g for 2 min. Supernatant was discarded and the pellet was suspended into 40 μL of 70% formic acid (Sigma-Aldrich, St. Quentin Fallavier, France), followed by vortexing for 5 s after addition of 40 μL of acetonitrile (Carlo Erba, Val de Reuil, France). Prior to MALDI-TOF MS analysis, all samples were centrifuged again at 14,000 × g for 2 min.

2.3. Spectra acquisition

One microliter of the obtained suspension was deposited on the target slide and allowed to dry, in duplicate for each sample. Then, 1 μL of α-cyano-hydroxycinnamic acid matrix solution (CHCA; bioMérieux, Marcy l'Etoile, France) was added to each spot and allowed to dry for 15 min. Spectra were acquired using the VITEK MS system (bioMérieux, Marcy l'Etoile, France) equipped with the Launchpad V2.8.4 acquisition software. All spectra were acquired in linear positive ion extraction mode in a mass range from 2000 to 20,000 Da. Individual spectra were accumulated from 500 laser shots (100 profiles with 5 shots per profile) with the 'Auto-Quality' option activated. The system was calibrated externally with fresh cells of *Escherichia coli* ATCC 8739. Raw spectra were automatically processed by smoothing and peak detection procedures implemented in Launchpad acquisition software. After acquisition, individual spectra from each strain were considered separately.

2.4. Spectra quality control

Raw spectra were controlled for peak resolution, signal to noise ratio, and absolute signal intensity. Good quality spectra were then transformed into peak lists and for each species, dendrograms with new spectra (and old spectra for species already in the clinical FDA-cleared VITEK MS database) were analyzed to assess intra-specific similarity and to detect any doubtful strains before integration of the spectra into the database.

2.5. Development of spectral database

As described by Girard et al. (2016), peak lists were binned and a log base scaling of the peak intensities was applied followed by a L1-normalization (Strubel et al., 2013). A predictive model was established for each species using the Advanced Spectra Classifier (ASC) algorithm developed by bioMérieux. This procedure provided a specific weighed bin matrix for each species and for identification, new spectra were compared to the bin weight matrix and the sum of matching bin weights was calculated and considered as an intermediate score. All species-specific scores were then transformed into multiclass probability estimates using a Gaussian based calibration procedure that took into account the score distributions within species compared to all other species (Strubel et al., 2013). A decision algorithm retained only significant matches and a single choice identification was obtained when only one species was retained. When more than one species was retained, a low discrimination result was proposed. In case more than 4 species were retained or if no significant match was found, it was considered as a non-identification result.

2.6. Optimization and performance evaluation of the database

A 5-fold cross validation was used to optimize the VITEK MS database and to assess how accurately it would perform on independent new spectra. The spectral data were randomly split into 5 subsets. One round of cross-validation involved a learning phase on 4 subsets, and validated the identification performance on the remaining subset. Five rounds of cross-validation were performed by permutation of the subsets. The results were combined across rounds to get an estimation of identification performance. A correct identification was defined when the same identification occurred between cross-validation result and the reference identification. Low discrimination results were considered as correct if the expected identification was included in the matches. A misidentification was defined as discordant identification between the cross-validation result and the reference identification.

2.7. Evaluation of identification performances

The database was challenged with 73 external strains corresponding to 276 spectra that met the quality criteria described above, in order to assess whether correct identification could be achieved for the species claimed in the database and whether no identification was provided for the species absent from the database.

3. Results and discussion

3.1. Selection of spectra for the reference database

A total of 6477 spectra and 618 strains were retained for database construction. For each strain, spectra were acquired on at least 3 different culture media and 2 different incubation times (2 and 8 days). Species with great relevance were represented by 5–10 strains whereas rare species were represented by a small number of strains. The average number of strains and spectra per species was 5 and 50, respectively.

3.2. Effects of incubation time and growth medium on spectral similarity

Concerning the effects of incubation time and growth medium on spectral classification, two different cases were observed. For most of the tested species, all spectra derived from a same strain clustered together according to the medium used for growth without any separation according to incubation time. This result indicated that spectra from a same species differed mainly according to the strains used to build the database and that the effects of growth medium and incubation time were less important than that of intra-species diversity.

For certain species such as *Aspergillus flavus*, *Aureobasidium*

Table 2
Species used for external validation and their strain collection and Genbank accession numbers.

Strain number	Name	Genbank accession number
UBOCC-A-101330	*Absidia glauca*	MH102058
3a18a	*Acremonium* sp.	KX928832.1
UBOCC-A-101046	*Alternaria brassicicola*	MH102059
UBOCC-A-101055	*Aspergillus clavatus*	MH122641
UBOCC-A-101060	*Aspergillus flavus*	KF225050.1
UBOCC-A-106016	*Aspergillus fumigatus*	MH122642
UBOCC-A-101054	*Aspergillus intermedius*	MH122643
UBOCC-A-110208	*Aspergillus nidulans*	KF499566
UBOCC-A-112119	*Aspergillus niger*	MH122644
UBOCC-A-111044	*Aspergillus tamarii*	MH122645
UBOCC-A-101082	*Aspergillus versicolor*	MH122646
UBOCC-A-111050	*Aspergillus wentii*	MH122647
UBOCC-A-111234	*Aureobasidium pullulans*	MH102060
UBOCC-A-110185	*Chaetomium globosum*	MH102061
3a14a	*Cladosporium cladosporioides*	KX928854.1
12a1	*Cladosporium halotolerans*	KX928934.1
12a17	*Cladosporium oxysporum*	KX928928.1
12a3a	*Cladosporium ramotenellum*	KX928855.1
UBOCC-A-101115	*Cladosporium sphaerospermum*	KJ596622.1
5i6	*Cladosporium sphaerospermum*	KX928840.1
1i2a	*Didymella glomerata*	KX928948.1
1i14	*Didymella heteroderae*	KX928949.1
1f11c	*Didymella pinodella*	KX928846.1
UBOCC-A-101137	*Fusarium avenaceum*	MH122636
UBOCC-A-109124	*Fusarium culmorum*	JF278586.1
UBOCC-A-109101	*Fusarium delphinoides*	EU926311.1
ATCC 20273	*Fusarium graminearum*	MH122637
1i17b	*Fusarium merismoides*	KX928848.1
UBOCC-A-101151	*Fusarium oxysporum*	MH122638
UBOCC-A-109112	*Fusarium poae*	JF278578.1
UBOCC-A-101164	*Fusarium solani*	MH122639
UBOCC-A-109014	*Fusarium sporotrichioides*	MH122640
UBOCC-A-216001	*Geotrichum candidum*	KX928847.1
UBOCC-A-109155	*Mucor racemosus*	JF723961.2
5f24	*Mucor circinelloides*	KX928835.1
UBOCC-A-108087	*Mucor fuscus*	KX928837.1
UBOCC-A-102007	*Mucor racemosus*	MH102062
5i3	*Mucor racemosus*	KX928839.1
CBS 133.37	*Paecilomyces niveus*	FJ390000.1
UBOCC-A-103043	*Paecilomyces variotii*	MH122648
1i10	*Penicillium adametzioides*	KX928888.1
5i7	*Penicillium antarticum*	KX928902.1
UBOCC-A-108092	*Penicillium aurantiogriseum*	NR_121247.1
5i16	*Penicillium bialowiezense*	KX928944.1
5i14	*Penicillium brevicompactum*	KX928920.1
UBOCC-A-110150	*Penicillium camemberti*	MH122649
CBS 112297	*Penicillium carneum*	AY674386.1
UBOCC-A-101400	*Penicillium chrysogenum*	KF225057.1
3a1a	*Penicillium chrysogenum*	KX928918.1
5i5	*Penicillium commune*	KX928952.1
UBOCC-A-110017	*Penicillium corylophilum*	KF225058.1
1i7b	*Penicillium dierckxii*	KX928892.1
UBOCC-A-111030	*Penicillium digitatum*	MH122650
5i1	*Penicillium discolor*	KX928906.1
12a3b	*Penicillium echinulatum*	KX928926.1
LCP 07.5414	*Penicillium expansum*	MH122651
UBOCC-A-108106	*Penicillium glabrum*	KF225079.1
12a4b	*Penicillium glabrum*	KX928905.1
UBOCC-A-110039	*Penicillium granulatum*	MH122652
5i4	*Penicillium nalgiovense*	KX928943.1
5i9	*Penicillium nordicum*	KX928912.1
5i25	*Penicillium palitans*	KX928947.1
CBS 464.95	*Penicillium paneum*	HQ442324.1
UBOCC-A-101449	*Penicillium roqueforti*	KM503582.1
5f23 (−3)	*Penicillium roqueforti*	KX928910.1
5i11	*Penicillium solitum*	KX928946.1
12a5c	*Penicillium spathulatum*	KX928913.1
UBOCC-A-114054	*Purpureocillium lilacinum*	KX928845.1
12a9	*Stereum* sp.	KX928844.1
5i8	*Thamnidium elegans*	MH113155
UBOCC-A-101290	*Trichoderma longibrachiatum*	MH102063
UBOCC-A-101312	*Verticillium dahliae*	MH102064
UBOCC-A-101325	*Wallemia sebi*	MH102065

pullulans and *Penicillium expansum*, a small effect of incubation time and growth medium was observed as shown in Fig. 1 for *P. expansum*. Indeed, for these species, different groups of spectra corresponding to the same medium (i.e. SDA or other media) and incubation time clustered together independently of the strain used (Fig. 1). This spectral grouping by medium and incubation time could be explained by the morphological changes that occurred as the culture aged, the main change being spore production. Consistent with this hypothesis, we observed that in these species, sporulation was strongly affected by incubation time and that sporulation was less important on SDA than for the other tested media (PDA, MEA and YGC agar). Despite these observations, identification performances were not affected as 100% of *P. expansum* spectra were correctly identified during cross-validation.

Two other media commonly used for food borne fungi cultivation were also tested, i.e., DG 18 and DRBC but were not chosen for database construction. Indeed, spectra acquired from closely related fungal species grown on these media could not be discriminated and a large proportion of spectra did not reach the chosen quality criteria (data not shown). This could be explained by the composition of these media which did not allow an optimal development of all fungi. Indeed, both media contain dichloran which is known to slow down mold growth (Henson, 1981) while DG18, a selective medium for xerophilic molds, has a low water activity and is therefore not adapted for growth of less xerotolerant fungi.

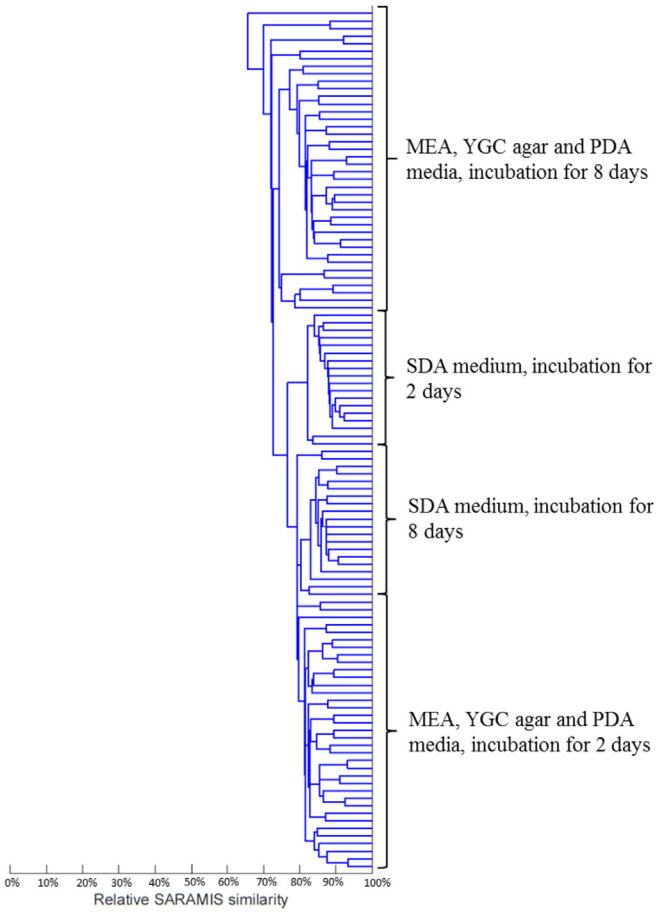

Fig. 1. Dendrogram showing spectral similarity of different strains belonging to *Penicillium expansum* cultivated on different growth media and for different incubation times.

There is little information about the impact of growth media on spectral similarity of fungi as most studies dealing with MALDI-TOF MS fungal identification were performed using only one cultivation medium. Concerning the effect of incubation time, several studies highlighted its impact on MALDI-TOF MS spectra classification. Alanio et al. (2011) observed differences between spectra of *Aspergillus* species obtained from young (<2 days of growth) and older thalli (4–10 days of growth) and developed their database with spectra from both young and mature thalli for more robustness as performed in the present study.

3.3. Performance evaluation by cross-validation

Global performance of the database was estimated by cross-validation. Overall, 94.12% of spectra were correctly identified to the species-level, 4.49% of spectra were not identified and 1.39% of spectra were discordant, meaning that they were incorrectly identified. Moreover, for species previously included into the VITEK MS database (e.g. *Aspergillus westerdijkiae* or *Epicoccum nigrum*), the new added spectra of the present study had no negative impact on the identification performances. Interestingly, Buskirk et al. (2011) argued that darkly-pigmented species such as *Aspergillus niger* had inhibitory effects on MALDI-TOF mass spectra analysis due to the presence of fungal melanin. However, in the present study, we did not observe such an impact on identification performances as more than 90% of *A. niger* spectra were correctly identified during cross-validation (Table 3).

Overall, detailed analysis of results (Table 3) showed that spectra from 113 out of the 136 species integrated into the database yielded an overall correct identification percentage above 90% with less than 10% spectra with low discrimination except for 7 species, i.e. *Aspergillus lactticoffeatus*, *Cladosporium oxysporum*, *Microdochium majus*, *Mucor hiemalis*, *Penicillium camemberti*, *Penicillium carneum* and *Trichiderma viride*. Noteworthy, most of these 113 species e.g. *Aspergillus flavus*, *Cladosporium halotolerans* or *Penicillium citrinum* had no discordant spectra and less than 5% unidentified spectra even within genus for which a large species diversity and closely related species were represented in the database e.g. *Penicillium* and *Aspergillus* spp. Spectra of the 23 remaining species had levels of correct identification <90% ranging from 42.9% to 89.8% for *Fusarium acuminatum* and *Cladosporium cladosporioides,* respectively. Not surprisingly, most species for which a low overall correct identification was achieved, were closely related with another species also represented in the database or belonged to species complex. For example, *Penicillium bialowiezense* spectra were not well discriminated from those of *Penicillium brevicompactum* and it is well established that both species are closely related and share similar colony and micromorphological characteristics (Samson et al., 2002). It was also the case for *Aspergillus foetidus* and *Aspergillus lacticoffeatus* for which a high percentage of discordant spectra (~39%) and low discrimination spectra (~37%) were obtained, respectively. These species are members of the *A. niger* clade together with *A. niger* and *Aspergillus tubingensis* and were misclassified with *A. niger*. Varga et al. (2011), based on sequence data and extrolite production, considered *Aspergillus foetidus* and *Aspergillus lacticoffeatus* as synonyms of *A. niger*. Interestingly, in Fig. 2 showing spectra similarity between these species, each species formed separated clusters except for *A. niger* and *A. lacticoffeatus*, thus only confirming partly results of Varga et al. (2011) as *A. foetidus* spectra were separated from those of *A. niger* and *A. lacticoffeatus*. Within *Fusarium* spp., identification percentages (<60%) were observed for *Fusarium acuminatum* and *Fusarium avenaceum* spectra, which are also closely related to *Fusarium arthrosporioides* and *Fusarium tricinctum* (Stakheev et al., 2016). When comparing spectra similarity, two distinct clusters could be

Table 3
Performance evaluation by cross-validation results.

Species	Overall correct (%)[b]	Single choice (%)	Low discrimination (%)	No identification (%)	Discordant (%)
Actinomucor elegans[a]	100	100	0	0	0
Alternaria alternata	98.97	98.97	0	1.03	0
Aspergillus arachidicola[a]	100	100	0	0	0
Aspergillus calidoustus	100	100	0	0	0
Aspergillus candidus	61.54	61.54	0	38.46	0
Aspergillus carbonarius[a]	93.18	93.18	0	6.82	0
Aspergillus chevalieri[a]	98.28	98.28	0	1.72	0
Aspergillus fennelliae[a]	100	100	0	0	0
Aspergillus flavus	99.59	99.59	0	0.41	0
Aspergillus foetidus[a]	44.44	25.93	18.52	16.67	38.89
Aspergillus fumigatus	99.59	99.59	0	0.41	0
Aspergillus glaucus	81.82	54.55	27.27	18.18	0
Aspergillus intermedius[a]	100	100	0	0	0
Aspergillus lacticoffeatus[a]	100	62.86	37.14	0	0
Aspergillus minisclerotigenes[a]	100	98.15	1.85	0	0
Aspergillus montevidensis[a]	100	98.48	1.52	0	0
Aspergillus nidulans	100	100	0	0	0
Aspergillus niger	90.74	87.04	3.7	6.17	3.09
Aspergillus novoparasiticus[a]	100	100	0	0	0
Aspergillus ochraceus	96.23	96.23	0	3.77	0
Aspergillus parasiticus[a]	87.5	87.5	0	12.5	0
Aspergillus penicilloides[a]	66.67	25	41.67	0	33.33
Aspergillus proliferans[a]	100	100	0	0	0
Aspergillus pseudoglaucus[a]	98.15	98.15	0	1.85	0
Aspergillus restrictus[a]	100	100	0	0	0
Aspergillus ruber[a]	96.97	93.94	3.03	3.03	0
Aspergillus sydowii	100	100	0	0	0
Aspergillus tamarii	100	100	0	0	0
Aspergillus terreus	99.54	99.54	0	0.46	0
Aspergillus thermomutatus	95.71	95.71	0	4.29	0
Aspergillus tubingensis	81.25	71.88	9.38	17.19	1.56
Aspergillus versicolor	95.4	95.4	0	4.6	0
Aspergillus wentii[a]	92.59	92.59	0	7.41	0
Aspergillus westerdijkiae	100	100	0	0	0
Aureobasidium pullulans	94.37	92.96	1.41	5.63	0
Botrytis cinerea[a]	100	100	0	0	0
Chaetomium globosum	100	100	0	0	0
Cladosporium cladosporioides	89.8	84.35	5.44	10.2	0
Cladosporium dominicanum[a]	97.06	97.06	0	2.94	0
Cladosporium fusiforme[a]	100	100	0	0	0
Cladosporium halotolerans[a]	100	100	0	0	0
Cladosporium herbarum[a]	77.08	77.08	0	5.21	17.71
Cladosporium langeronii[a]	94.59	94.59	0	5.41	0
Cladosporium oxysporum[a]	100	86.44	13.56	0	0
Cladosporium psychrotolerans[a]	100	100	0	0	0
Cladosporium ramotenellum[a]	100	100	0	0	0
Cladosporium sphaerospermum	100	100	0	0	0
Cladosporium velox[a]	100	100	0	0	0
Didymella glomerata[a]	83.93	64.29	19.64	14.29	1.79
Didymella pinodella[a]	55	50	5	0	45
Epicoccum nigrum	100	100	0	0	0
Fusarium acuminatum[a]	42.86	26.53	16.33	38.78	18.37
Fusarium arthrosporioides[a]	90.91	90.91	0	9.09	0
Fusarium avenaceum[a]	58.93	57.14	1.79	41.07	0
Fusarium cerealis[a]	100	100	0	0	0
Fusarium culmorum[a]	96.55	91.38	5.17	0	3.45
Fusarium domesticum[a]	100	100	0	0	0
Fusarium equiseti[a]	67.14	67.14	0	31.43	1.43
Fusarium graminearum[a]	100	100	0	0	0
Fusarium incarnatum[a]	83.33	76.67	6.67	13.33	3.33
Fusarium langsethiae[a]	100	100	0	0	0
Fusarium lateritium[a]	100	100	0	0	0
Fusarium oxysporum	97.76	97.01	0.75	1.49	0.75
Fusarium poae[a]	97.06	97.06	0	2.94	0
Fusarium proliferatum	97.22	96.3	0.93	2.78	0
Fusarium sambucinum[a]	100	100	0	0	0
Fusarium solani	96	96	0	4	0
Fusarium sporotrichioides[a]	98	94	4	2	0
Fusarium subglutinans[a]	86.67	80	6.67	13.33	0
Fusarium temperatum[a]	100	97.83	2.17	0	0
Fusarium tricinctum	95.45	92.42	3.03	1.52	3.03
Fusarium venenatum[a]	100	100	0	0	0
Fusarium verticillioides	71.01	71.01	0	26.09	2.9
Geotrichum candidum	98.72	98.72	0	1.28	0

(*continued on next page*)

Table 3 (*continued*)

Species	Overall correct (%)[b]	Single choice (%)	Low discrimination (%)	No identification (%)	Discordant (%)
Lasiodiplodia theobromae[a]	100	100	0	0	0
Lichtheimia corymbifera	100	100	0	0	0
Macrophomina phaesolina[a]	93.33	86.67	6.67	0	6.67
Microdochium majus[a]	100	83.33	16.67	0	0
Microdochium nivale[a]	100	100	0	0	0
Mucor circinelloides	95.83	93.75	2.08	4.17	0
Mucor fragilis[a]	100	97.06	2.94	0	0
Mucor hiemalis[a]	100	90.91	9.09	0	0
Mucor lanceolatus	97.56	97.56	0	0	2.44
Mucor plumbeus[a]	100	100	0	0	0
Mucor racemosus	100	100	0	0	0
Paecilomyces divaricatus[a]	100	100	0	0	0
Paecilomyces formosus[a]	98.41	98.41	0	1.59	0
Paecilomyces fulvus	93.06	93.06	0	6.94	0
Paecilomyces saturatus[a]	100	100	0	0	0
Paecilomyces variotii	98.6	98.6	0	1.4	0
Penicillium adametzioides[a]	100	100	0	0	0
Penicillium antarcticum[a]	100	100	0	0	0
Penicillium aurantiogriseum[a]	93.55	83.87	9.68	4.84	1.61
Penicillium bialowiezense[a]	87.72	85.96	1.75	12.28	0
Penicillium brevicompactum	98.48	93.18	5.3	1.52	0
Penicillium camemberti	95.65	77.39	18.26	0	4.35
Penicillium carneum[a]	100	80.85	19.15	0	0
Penicillium chermesinum[a]	91.67	91.67	0	8.33	0
Penicillium chrysogenum	96.18	96.18	0	2.55	1.27
Penicillium citreonigrum[a]	100	100	0	0	0
Penicillium citrinum	98.46	98.46	0	1.54	0
Penicillium commune[a]	62.35	42.35	20	12.94	24.71
Penicillium corylophilum[a]	100	100	0	0	0
Penicillium crustosum[a]	98.72	96.15	2.56	1.28	0
Penicillium decumbens	100	100	0	0	0
Penicillium dierckxii[a]	100	100	0	0	0
Penicillium digitatum[a]	100	100	0	0	0
Penicillium discolor[a]	79.55	77.27	2.27	0	20.45
Penicillium expansum	100	100	0	0	0
Penicillium fuscoglaucum[a]	84.62	76.92	7.69	0	15.38
Penicillium glabrum	100	100	0	0	0
Penicillium italicum	100	100	0	0	0
Penicillium nalgiovense[a]	96.43	96.43	0	3.57	0
Penicillium nordicum[a]	100	100	0	0	0
Penicillium oxalicum[a]	81.43	81.43	0	18.57	0
Penicillium palitans[a]	63.16	57.89	5.26	23.68	13.16
Penicillium paneum[a]	95.92	95.92	0	4.08	0
Penicillium polonicum[a]	94.64	92.86	1.79	5.36	0
Penicillium roqueforti	92.86	92.26	0.6	0.6	6.55
Penicillium salamii[a]	100	100	0	0	0
Penicillium simplicissimum[a]	100	100	0	0	0
Penicillium solitum[a]	100	100	0	0	0
Penicillium verrucosum[a]	93.48	93.48	0	6.52	0
Penicillium viridicatum[a]	65.63	65.63	0	34.38	0
Purpureocillium lilacinum	100	100	0	0	0
Rhizopus oryzae complex	92.86	89.29	3.57	7.14	0
Rhizopus stolonifer[a]	94.74	92.11	2.63	5.26	0
Scopulariopsis asperula[a]	100	100	0	0	0
Talaromyces bacillisporus[a]	98.28	98.28	0	1.72	0
Talaromyces macrosporus[a]	100	100	0	0	0
Trichoderma harzianum[a]	100	100	0	0	0
Trichoderma viride[a]	100	84.85	15.15	0	0
Trichothecium roseum[a]	96.67	96.67	0	3.33	0
Umbelopsis isabellina[a]	100	100	0	0	0
Wallemia sebi[a]	100	100	0	0	0

[a] Species not previously represented in the VITEK MS fungal database.
[b] Single choice percentage corresponds to spectra identified to the correct species, low discrimination percentage corresponds to spectra which matched with several species including the correct one while overall correct percentage is the addition of single choice and low discrimination percentages.

observed i.e., one containing *F. acuminatum* and *F. tricinctum* spectra and another one containing *F. avenaceum* and *F. arthrosporoides* spectra (data not shown). Among *Penicillium* spp., low levels of correctly identified spectra were achieved for members of the *Penicillium camemberti*/*Penicillium commune* species complex, i.e., *P. commune*, *Penicillium fuscoglaucum* and *Penicillium palitans*. Indeed, all these species including *P. camemberti* are also phylogenetically closely related (Giraud et al., 2010). While *P. camemberti* and *P. commune* only differ slightly in their morphological characteristics as they produce respectively white and green conidia, it has been shown that they cannot be distinguished using molecular data (Giraud et al., 2010). Giraud et al. (2010) also proposed to re-introduce the old name *P. fuscoglaucum* (previously synonymized with *P. commune*) based on tubulin gene and PC4 microsatellite genealogies while it was shown that *P. palitans* was closely related but distinguishable from

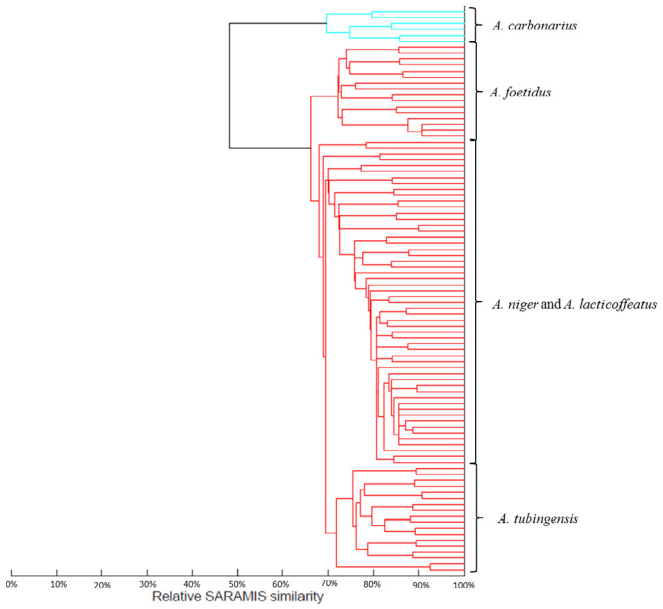

Fig. 2. Dendrogram showing spectral similarity of different strains belonging to 4 species from the *A. niger* clade and *A. carbonarius*.

P. camemberti/*P.commune*. Based on these data and the present results, it may not be possible to identify these closely related species accurately using MALDI-TOF and it would be necessary to group these species into a complex as it was already done for closely related bacterial species such as those from the *Mycobacterium tuberculosis* complex (Girard et al., 2016). Interestingly, we were able to accurately identify all members of the *Cladosporium sphaerospermum* complex, e.g. C. *dominicanum*, *C. psychrotolerans*, *C. halotolerans*, *C. sphaerospermum*, *C. fusiforme*, *C. velox* and *C. langeronii* (Table 3) as these species harboured quite different MALDI-TOF spectra (Fig. 3).

This method appears as powerful as the molecular gold standard to identify most of the species we implemented in the database, even for closely related species. Indeed, as underlined recently by Normand et al. (2013), the analysis of a high number of subcultures from each strain and of strains representing each species are key to improve the effectiveness of spectral libraries for fungal identification. However, this method may also have limitations for certain species complex.

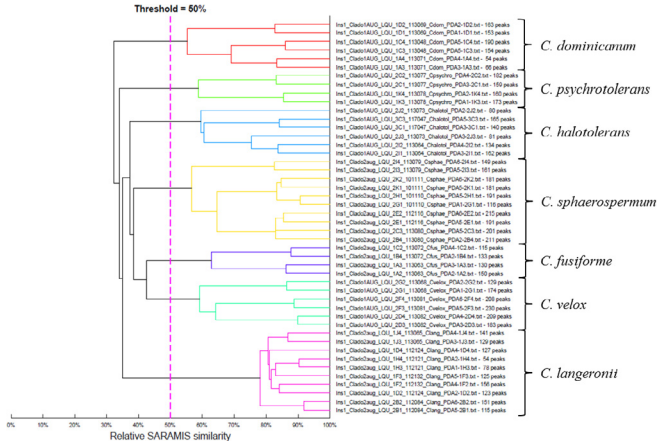

Fig. 3. Dendrogram showing spectral similarity of different strains and species belonging to the *Cladosporium sphaerospermum* complex.

Although these results were very promising, this first cross-validation could only estimate the database performances and give a first idea on how the different species behaved. In order to go further in the evaluation and validation of the method performances, we then tested external strains not used to build the database, in order to assess whether they could be correctly identified or not.

3.4. Database validation with external isolates

The database was challenged with external isolates to evaluate its performances. Of the 73 external strains used, corresponding to 19 genera and 67 species, 52 strains belonged to species represented in the database and 15 belonged to species absent from it. Depending on the analyzed strains, different cases were observed. For 69 out of 73 strains, spectra acquired from the same strain showed identical results (either a good, bad or no identification) (Table 4). Exceptions were *Penicillium aurantiogriseum* UBOCC-A-108092 and *Penicillium expansum* UBOCC-A-108103 for which spectra were either correctly or not identified, and *Penicillium granulatum* UBOCC-A-110039, *Penicillium spathulatum* 12a5c and *Verticillium dahliae* UBOCC-A-101312 for which spectra were either incorrectly identified or not identified. There were not any strains for which spectra were assigned to two or more different species as single choice.

For species integrated into the database, 89.45% of spectra were correctly identified to the species-level, 4.13% of spectra were not identified and 6.42% of spectra were erroneously identified (discordant). The misidentification corresponded to spectra of *Cladosporium oxysporum* identified as *Cladosporium cladosporioides* complex; *P. commune* spectra identified as *P. camemberti*; *P. palitans* spectra identified as *P. aurantiogriseum* and *Didymella pinodella* spectra identified as *Didymella glomerata*. It can be emphasized that spectra which were misidentified corresponded to species from the same genus and in the case of *P. commune* and *P. camemberti,* for undistinguishable species based on molecular data. For the 15 species not represented into the database, 70.69% of spectra were not identified and 29.31% of spectra were identified to the wrong species. As shown in Table 4, most of the misidentified spectra were assigned to species of the same genus.

Although some of the tested spectra for this external validation were not correctly identified, their assignment at the genus level was correct. For the species present in the database, all spectra acquired from 52 out of 58 strains were correctly assigned. Since they were obtained with a non-optimized database, these results are promising and should improve as the database is further optimized. Optimization could consist in grouping closely related species into species complex as mentioned above, adding spectra from other species and new strains for the species already present in the database.

In comparison to the present study, the three most clinically comprehensive mold databases were developed by Lau et al. (2013), Gautier et al. (2014) and Becker et al. (2015), with respectively 152, 347 and 525 claimed fungal species. When challenged with external isolates, identification performances of these databases respectively yielded 88.9%, 98.8% and 84% of correctly identified spectra. The levels of correctly identified spectra obtained by Lau et al. (2013) and Becker et al. (2015) were close but below those obtained in the present study, with 89.45% of correctly identified external spectra. As mentioned previously, these results demonstrate the good reliability of the spectral database covering 136 species for identification of food spoilage fungi.

4. Conclusion

MALDI-TOF MS appears to be a rapid and reliable tool for the

Table 4
Detailed results for the set of external strains used to evaluate the database performances.

	Species (number of strains)	Number of spectra	Number of correctly identified spectra	Number of non-identified spectra	Number of misidentified spectra
Species present in the database	*Aspergillus flavus* (1)	4	4	0	0
	Aspergillus fumigatus (1)	4	4	0	0
	Aspergillus intermedius (1)	4	4	0	0
	Aspergillus nidulans (1)	4	4	0	0
	Aspergillus niger (1)	4	4	0	0
	Aspergillus tamarii (1)	4	4	0	0
	Aspergillus versicolor (1)	4	4	0	0
	Aspergillus wentii (1)	4	4	0	0
	Aureobasidium pullulans (1)	4	4	0	0
	Chaetomium globosum (1)	2	0	2	0
	Cladosporium cladosporioides (1)	4	4	0	0
	Cladosporium halotolerans (1)	4	4	0	0
	Cladosporium oxysporum (1)	4	0	0	4 (*C. cladosporioides* complex)
	Cladosporium ramotenellum (1)	4	4	0	0
	Cladosporium sphaerospermum (2)	6	6	0	0
	Didymella glomerata (1)	4	4	0	0
	Didymella pinodella (1)	2	0	0	2 (*D. glomerata*)
	Fusarium avenaceum (1)	2	0	2	0
	Fusarium culmorum (1)	4	4	0	0
	Fusarium graminearum (1)	2	2	0	0
	Fusarium oxysporum (1)	4	4	0	0
	Fusarium poae (1)	4	4	0	0
	Fusarium solani (1)	2	2	0	0
	Fusarium sporotrichioides (1)	4	4	0	0
	Geotrichum candidum (1)	4	4	0	0
	Mucor circinelloides (1)	4	4	0	0
	Mucor racemosus (3)	12	12	0	0
	Paecilomyces variotii (1)	4	4	0	0
	Penicillium adametzioides (1)	4	4	0	0
	Penicillium antarcticum (1)	4	4	0	0
	Penicillium aurantiogriseum (1)	4	2	2	0
	Penicillium bialowiezense (1)	4	4	0	0
	Penicillium brevicompactum (1)	4	4	0	0
	Penicillium camemberti (1)	4	4	0	0
	Penicillium carneum (1)	4	4	0	0
	Penicillium chrysogenum (2)	8	8	0	0
	Penicillium commune (1)	4	0	0	4 (*P. camemberti*)
	Penicillium corylophilum (1)	4	4	0	0
	Penicillium digitatum (1)	4	4	0	0
	Penicillium discolor (1)	4	4	0	0
	Penicillium expansum (1)	4	3	1	0
	Penicillium dierckxii (1)	4	4	0	0
	Penicillium glabrum (2)	8	8	0	0
	Penicillium nalgiovense (1)	4	4	0	0
	Penicillium nordicum (1)	2	2	0	0
	Penicillium palitans (1)	4	0	0	4 (*P. aurantiogriseum*)
	Penicillium paneum (1)	4	4	0	0
	Penicillium roqueforti (2)	8	8	0	0
	Penicillium solitum (1)	4	4	0	0
	Purpureocillium lilacinum (1)	4	4	0	0
	Trichoderma longibrachiatum (1)	4	4	0	0
	Wallemia sebi (1)	4	4	0	0
Species absent from the database	*Absidia glauca* (1)	4	–	4	0
	Acremonium sp. (1)	2	–	2	0
	Alternaria brassicicola (1)	4	–	0	4 (*A. alternata*)
	Aspergillus clavatus (1)	4	–	4	0
	Didymella heteroderae (1)	4	–	0	4 (*D. glomerata*)
	Fusarium delphinoides (1)	4	–	4	0
	Fusarium merismoides (1)	4	–	4	0
	Mucor fuscus (1)	4	–	4	0
	Paecilomyces niveus (1)	4	–	0	4 (*P. fulvus*)
	Penicillium echinulatum (1)	4	–	4	0
	Penicillium granulatum (1)	4	–	2	2 (*Rhizopus arrizhus* complex)
	Penicillium spathulatum (1)	4	–	2	2 (*P. brevicompactum*)

Table 4 (*continued*)

	Species (number of strains)	Number of spectra	Number of correctly identified spectra	Number of non-identified spectra	Number of misidentified spectra
	Stereum sp. (1)	4	–	4	0
	Thamnidium elegans (1)	4	–	4	0
	Verticillium dahliae (1)	4	–	3	1 (*Fusarium solani* complex)

identification of filamentous fungi. Using the present standardized extraction protocol, it is rapid and easy to implement as compared to phenotypic and genotypic methods. Moreover, this method is robust enough to allow the use of several different culture media and incubation time for identification and to our best knowledge, this database is the most comprehensive one developed for the identification of food spoilage fungi. It appeared as powerful as DNA sequencing to identify most of the species we implemented in the database, even for closely related species. However, it also had limitations for certain species complex. All together, these results also emphasize the need to use well characterized strains to build a spectral database for identification and to identify its potential limits. With the implementation of this new database and the constant evolution of fungal taxonomy and phylogeny (Houbraken and Samson, 2017), one challenge will be to keep the database taxonomy updated and to regularly add new species and new strains for species already represented in the database. Another challenge to address will be to assess whether MALDI-TOF MS can be applied for intra-specific differentiation or species complex resolution as already shown for certain yeast (Stübiger et al., 2016) and bacterial (Dieckmann et al., 2008) species.

Conflicts of interest

LQ, VG, SA, BC, VM and AVB are employees of bioMérieux, a company developing and selling in vitro diagnostic assays including the VITEK MS used in this study.

Acknowledgements

This work was done as part of a CIFRE PhD funded by bioMérieux and the French Association for Research and Technology (ANRT) [Convention #2015/0821] in collaboration with the LUBEM laboratory.

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