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Identification et typage de champignons filamenteux et de levures d'intérêt alimentaire par spectrométrie de masse MALDI-TOF

Nolwenn Rolland

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Par

Nolwenn ROLLAND

Identification et typage de champignons filamenteux et de levures d'intérêt alimentaire par spectrométrie de masse MALDI-TOF

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Non quia difficilia sunt non audemus, sed quia non audemus difficilia sunt.

- Sénèque -

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Lexique des abréviations

AFNOR : Association Française de NORmalisation

AFSSA : Agence Française de Sécurité Sanitaire des Aliments

ANRT : Association Nationale de la Recherche et de la Technologie

APA : Accès et Partage des Avantages découlant de l'utilisation des ressources génétiques et des connaissances traditionnelles associées

ASC : Advanced Spectra Classifier

ATCC : American Type Culture Collection

a_w : Activity of Water

BD : Becton Dickinson

BSC : Biological Species Concept

BVP : Boulangerie Viennoiserie Pâtisserie

CHCA : α -Cyano-4-HydroxyCinnamic Acid

CIFRE : Convention Industrielle de Formation par la REcherche

DNA : DeoxyriboNucleic Acid

DG18 : Dichloran Glycérol

DRBC : Dichloran Rose Bengale Chloramphénicol

ED EGAAL : Ecole Doctorale Ecologie, Géosciences, Agronomie, Alimentation

eMLG : MultiLocus Genotypes

EQUASA : Etudes en QUALité et Sécurité dans l'Agroalimentaire

ESC : Ecological Species Concept

ESIAB : Ecole Supérieure d'Ingénieur en Agroalimentaire de Bretagne atlantique

FAO : Food and Agriculture Organization

FDA : Food and Drug Administration

GCPSR : Genealogical Concordance Phylogenetic Species Recognition

Hexp : expected heterozygosity

Ia : Index of association

INRAE : Institut National de Recherche pour l'Agriculture, l'alimentation et l'Environnement

ITS : Internal Transcribed Spacer

JS : Journées Scientifiques

KB : Knowledge Base

LUBEM : Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne

MALDI-TOF : Matrix-Assisted Laser Desorption / Ionization - Time-Of-Flight

MCMC : Markov Chain Monte Carlo

MEA : Malt Extract Agar

MLG : MultiLocus Genotypes

MS : Mass Spectrometry

MSC : Morphological Species Concept

NGS : Next-Generation Sequencing

NMDS : Non-Metric Multidimensional Scaling

OGA : Oxytetracycline Glucose Agar base

PCR : Polymerase Chain Reaction

PCoA : Principal Coordinate Analysis

PDA : Potato Dextrose Agar

PSC : Phylogenetic Species Concept

r_{barD} : standardized index of association

RNA : RiboNucleic Acid

SDA : Sabouraud Dextrose Agar

SDCN : Sabouraud Dextrose Chloramphenicol Neutralizers

SE : Standard Error

SFM : Société Française de Microbiologie

SNP-seq : Single Nucleotide Polymorphisms sequencing

SSRs : Simple Sequence Repeats

SSR-seq : Simple Sequence Repeats sequencing

TFU : Thallus Forming Unit

t-SNE : t – Stochastic Neighbor Embedding

UBOCC : Université de Bretagne Occidentale Culture Collection

YGC : Yeast Glucose Chloramphenicol agar

Avant-propos

Cette thèse CIFRE s'inscrit dans la continuité d'un précédent travail doctoral, issu d'une collaboration entre bioMérieux et le LUBEM (projet SPECTROFUN – thèse CIFRE 2015-2018), ayant principalement porté sur le développement de la spectrométrie de masse Matrix-Assisted Laser Desorption / Ionization - Time-Of-Flight (MALDI-TOF) pour l'identification des champignons filamenteux d'intérêt alimentaire (Quéro, 2018). Dans le cadre de ce projet, les performances d'identification de la base de données ainsi construite ont par la suite été évaluées par validation croisée et validation externe avec respectivement ~95% et ~90% de spectres correctement identifiés. Ces méthodes permettent d'estimer avec précision les performances grâce à un modèle prédictif appliqué sur un jeu de données, elles seront détaillées plus avant dans le manuscrit. Par la suite, une preuve de concept avait pu être établie dans le cadre de l'évaluation du pouvoir discriminant de cette technique. En effet, elle a été appliquée à la résolution de complexes d'espèces, incluant des espèces cryptiques, et au typage (Quéro et al., 2020). Les résultats de cette étude démontrent les capacités de différenciation du MALDI-TOF au sein de la section *Flavi* d'*Aspergillus* et dans le cas d'isolats de *Penicillium roqueforti* appartenant à trois populations génétiquement différenciées. D'une part, 99% des spectres ont été correctement attribués à l'espèce lors de l'analyse par validation croisée des spectres d'*Aspergillus* section *Flavi*. D'autre part, lors des validations croisée et externe, 94% et 95% des spectres de *Penicillium roqueforti* ont été correctement attribués à leur population génétique d'origine. Or, les résultats sont analogues à ceux résultant d'une approche polyphasique fondée sur l'analyse combinée de 3 gènes (Frisvad et al., 2019). Ces résultats probants ont donc amené à la reconduction d'un projet de thèse afin de poursuivre l'extension de cette base de données commerciale mais également d'explorer plus avant la capacité de discrimination du MALDI-TOF MS dans un contexte industriel concret.

Ce manuscrit constitue un exposé des données collectées lors de la thèse CIFRE subséquente aux travaux doctoraux précédemment exposés. Il a été articulé comme suit :

- La première section est un état de l'art. Il traite notamment de l'impact et des risques liés au développement fongique dans un contexte agro-alimentaire, des difficultés et des enjeux de l'identification de ces organismes ainsi que des approches employées pour cela, telle que la spectrométrie de masse MALDI-TOF. Cette synthèse bibliographique est constituée d'un

chapitre d'ouvrage intitulé « Identification of Mold Species and Species Complex from the Food Environment Using MALDI-TOF MS », paru en 2023, ainsi que des objectifs de thèse.

- La seconde section englobe l'ensemble des résultats recueillis au cours de cette thèse et est subdivisée en deux chapitres. Tout d'abord, le premier chapitre expose la construction et l'extension d'une base de données spectrales grâce à des espèces fongiques, levures et moisissures, majoritairement retrouvées dans le secteur agro-alimentaire. Les validations croisée et externe de la base de données spectrales développée sont également présentées. Ensuite, le second chapitre porte sur la diversité et les voies de contamination de moisissures d'altération de produits de boulangerie en utilisant la spectrométrie de masse MALDI-TOF et des approches moléculaires dans un contexte industriel (usine de Boulangerie Viennoiserie Pâtisserie (BVP)). D'une part, il y est exposé l'étude de la diversité au niveau inter- et intraspécifique des champignons présents que ce soit dans l'environnement ou les produits eux-mêmes. D'autre part, les résultats obtenus par génotypage et spectrométrie de masse MALDI-TOF sont confrontés afin d'évaluer l'utilisation de cet outil pour le typage (source-tracking) dans un contexte industriel.

- La troisième section présente une discussion générale des problématiques abordées, des questionnements soulevés, des résultats obtenus et des perspectives émergeant au terme de cette thèse.

- La quatrième et dernière section fait état des valorisations liées au projet de thèse, c'est-à-dire les communications orales et posters réalisées ainsi que les publications écrites.

Introduction bibliographique

Chapitre d'ouvrage : Identification of Mold Species and Species complex from the Food Environment Using MALDI-TOF MS

Girard V., Monnin V., **Rolland N.**, Mounier J., Jany J.-L. (2023) Identification of Mold Species and Species Complex from the Food Environment using MALDI-TOF MS. In: Microbiological Identification using MALDI-TOF and Tandem Mass Spectrometry: Industrial and Environmental Applications. Shah A.H. et al (Ed.). Wiley, 497-514.

I. Fungal taxonomy

1. Defining what is a fungal species

Fungal taxonomy is based on a classification which, in accordance with the International Code of Nomenclature for algae, fungi and plants (Turland et al., 2018), includes seven primary ranks, i.e. from the higher to the lower hierarchical rank - kingdom, phylum, class, order, family, genus and species - as well as secondary ranks (e.g. variety and form at the subspecies level) and further ranks (e.g. subforma). Among these different hierarchical ranks, species is considered as the fundamental unit to which evolutionary changes take place (Huxley, 1942). Therefore, species will evolve unique characteristics over time, and although intraspecific variability does occur, global functional properties such as growth parameters or metabolite production are usually assigned to the fundamental unit, i.e. at the species level.

Several definitions of a species have been proposed from John Ray (1686, in Cain, 1999) through Linnaeus (1797), Darwin (1859) or Mayr (1942). However, most of these definitions require the existence of sexual reproduction to define species and do not apply, for instance, to asexual fungal groups. More recent definitions, such as the one proposed by De Queiroz (1998), are more widely accepted. For this author, species correspond to « segments of evolutionary lineages that evolve independently from one another ». The process that leads two lineages to evolve independently from an initial ancestral population is speciation. Speciation takes place when reproductive isolation occurs between population, when the gene flow between populations is precluded (for reviews see: Natvig and May, 1996; Burnett and Burnett, 2003; Kohn, 2005; Giraud *et al.*, 2008; Chethana *et al.*, 2021). Most often, speciation is the result of ecologically based divergent selection. This may arise from differences in ecological niches which include abiotic and biotic factors, intra- and interspecific interactions, artificial selection (domestication) and/or sexual selection. Many forms of reproductive isolation can preclude gene flow between populations, including both pre- and post-zygotic barriers. When the genes under divergent selection and the genes causing reproductive isolation differ, reproductive isolation in sympatry evolves by indirect selection, i.e. linkage disequilibrium of pleiotropy (Rundle and Nosil, 2005).

2. Fungal speciation within a food context

Compared to other habitats, food matrices may represent richer, less competitive and more stable environments (Gibbons and Rinker, 2015) in which retained allelic combinations in the fungal population may differ drastically from the ones of other habitats. Species mainly encountered in food environments or even only encountered in food matrices might correspond to food-adapted species. For example, the cheese-colonizing but not inoculated species *Mucor lanceolatus*, which plays a role in Tomme or Saint-Nectaire ripening (Hermet et al., 2012) and exhibits traits beneficial for cheese colonization (Morin-Sardin et al., 2016), might be considered as a cheese-adapted species (Lebreton et al., 2020).

A specific case of speciation through adaptive divergence in response to artificial selection is domestication (Gladieux et al., 2014). This refers to the genetic modification of a species by breeding it in isolation from its ancestral population in an effort to enhance its utility to humans (Gibbons and Rinker, 2015); for example, *Aspergillus oryzae*, *Monascus purpureus*, *Penicillium camemberti*, *Penicillium nalgiovense* or *Saccharomyces cerevisiae* inoculated as adjunct cultures for food fermentations (Gladieux et al., 2014) are considered as domesticated species. One expects that domesticated fungi have been selected (by humans) for beneficial traits conferred by certain alleles (implying a global relaxation of selective constraints but a positive selection on the genes corresponding to alleles conferring beneficial traits in a food production context) and have undergone several rounds of population bottlenecks (genetic drift) (Gibbons and Rinker, 2015).

3. Delimiting species

Beyond the definition of a fungal species and the understanding of the evolutionary forces that drive speciation, it is important to consider species criteria that allow for species recognition. Indeed, what is called species concept often corresponds to species criteria to detect a species (Giraud et al., 2008). The species definition describes entities, whereas species concept corresponds to criteria to delimit species boundaries (Chethana et al., 2021) and, as

proposed by Taylor *et al.* (2000), the term species concept should be replaced for operational species criteria. These operational species criteria are: the biological species concept (BSC), which emphasizes reproductive isolation; the morphological species concept (MSC), which emphasizes morphological divergences; the ecological species concept (ESC), which emphasizes adaptation to a specific niche; and the phylogenetic species concept (PSC), based on nucleotide divergence analysis (Giraud *et al.*, 2008). These different species criteria may be discordant and importantly species recognition using these criteria eventually lead to a species hypothesis: species recognition is putative. Indeed, as highlighted by Giraud *et al.*, (2008), speciation is a temporally extended process during which the phenomena used for species recognition by BSC, MSC, ESC or PSC, i.e. reproductive isolation, morphological divergence, niche adaptation and nucleotide divergence, do not necessarily occur and do not necessarily appear in a conserved chronology during the course of the speciation process.

Most often than not, polyphasic approaches are conducted and recent descriptions of new species usually rely on the simultaneous use of the different operational species criteria. If morphological observation, mating test or ecological niche assignment approaches rely on conserved methods, genetic divergence assessment through the PSC can be achieved using different approaches. All of them are based on sequence alignments pertaining to multiple fungal strains using different alignment algorithms followed by phylogenetic reconstructions. Sequence alignment and edition is of particular importance as phylogenetic reconstructions are often based on character-based methods (each nucleotide position being considered as a character) such as Maximum Likelihood or Bayesian Inference (Felsenstein and Felsenstein, 2004). The output of such pipelines is a consensus phylogenetic tree that may be accompanied by multiple single locus trees used to delineate the species boundaries within the consensus tree. The most used phylogenetic approach for PSC, the GCPSR (Genealogical Concordance Phylogenetic Species Recognition) has been described by Taylor *et al.* (2000). It uses multiple unlinked genes to detect the absence of genetic recombination between lineages and, thus, evolutionary independence of these lineages. The coalescent-based species delimitation which uses a more probabilistic approach can also be used. In the omic era, phylogenetic studies tend to be replaced by phylogenomic studies using not handfuls of genes but rather thousands of orthologous genes.

As highlighted earlier, species are hypothetical as the operational species criteria may fail in correctly detecting segments of evolutionary lineages that evolve independently from one another (de Queiroz, 1998). Sometimes, species delimitation is difficult, not possible or leads to incongruent data when considering different criteria. Therefore, some taxonomic entities may encompass different species with species boundaries that are not well defined. These groups are unofficially called species complex. They may correspond, for example, to entities that actually contain a number of "cryptic species", which are genetically distinct species erroneously classified as a single species due to at least superficial morphological similarity observed using the MSC. This is why the use of PSC has recently allowed mycologists to amend the fungal taxonomy by detecting species boundaries within several species complexes.

4. Foodborne fungi within the fungal tree of life

The fungal kingdom is estimated to encompass between 2.2 and 3.8 million species among which less than 10 % are named (Hawksworth and Lücking, 2017), and harbors considerable functional diversity, playing major roles as ecological actors as well as in industrial applications, in particular for food and feed productions. Fungal species are clustered within twelve phyla according to James *et al.*, (2020) or alternatively within 19 phyla according to Wijayawardene *et al.*, (2020). Foodborne fungal species mainly belong to the *Ascomycota* and *Mucoromycota* and, more particularly, to the *Mucorales* order within the *Mucoromycotina* subphylum. Some food contaminants (e.g. *Wallemia sebi*) are also encountered within the *Basidiomycota*. As illustrated by the fungal tree of life (Spatafora *et al.*, 2017), the *Mucoromycota* phylum diverged earlier than the dikarya, which encompass both *Ascomycota* and *Basidiomycota* phyla. Species pertaining to these taxonomic groups harbor morphological and ecological differences. Basically, most fungi within the *Mucorales* (*Mucoromycota*, *Mucoromycotina*, *Mucorales*) are characterized by coenocytic hyphae, sporangial asexual reproduction and zygospores as sexual spores (Spatafora *et al.*, 2017). Their ecological strategy has often been described as ruderal or R-selected (Cooke and Rayner, 1984). This ruderal strategy is associated with rapid growth, rapid commitment to reproduction, utilization of easily assimilable resources and non-persistence. Importantly, there are no mycotoxin producers

among the *Mucorales*. Dikarya is the only subkingdom of Fungi and comprises the phyla *Ascomycota* and *Basidiomycota*. Their hyphae are regularly septated and, at some point in their life cycle, the hyphal compartments delimited by the septa possess two genotypically distinct nuclei (Spatafora et al., 2017). Most foodborne fungi (either among the contaminants or among the adjunct cultures) belong to the *Ascomycetes*. The latter are characterized by a stress-tolerant ecological strategies (Cooke and Rayner, 1984) with usually high levels of conidia (asexual spore) production and strong capabilities in facing biotic and abiotic constraints. As such, numerous species within the *Ascomycota* can produce mycotoxins, which among other roles, may confer an advantage against competitors.

II. Impact of molds in food

Depending on the food context, molds can have desirable or undesirable impacts on food quality and safety. Indeed, filamentous fungi can colonize a very large diversity of food raw materials (fruits, vegetables etc.) and processed foods, and produce a large panel of enzymes which will lead to different metabolites and reaction products that allow them to increase their biomass. In most cases, fungal colonization of a given food substrate, is considered as undesirable because of its negative impact on food texture/structure, visual aspect (e.g. surface color) and flavor through, for example, production of volatile compounds and sometimes because of the presence of mycotoxins which are secondary metabolites toxic for human and animal health. During human history, the colonization by specific molds of certain food substrates has led to a favorable effect on food quality attributes by increasing the shelf-life and by improving or contributing to the development of (new) organoleptic properties of food products, which are termed as fermented foods (Nout, 2007).

1. Filamentous fungi in fermented foods

Filamentous fungi encountered in fermented foods are not, strictly speaking, fermentative organisms, fermentation being defined as an anaerobic process by which organisms generate energy, but they are tightly associated with a large number of fermented foods in which they make a significant contribution, in association or not with other microorganisms (i.e., yeasts, bacteria), to the improvement of their organoleptic properties. Originally born in the East, fermentation processes using filamentous fungi have progressively spread to Western countries, mainly in food products of animal origin (i.e. fermented dairy and meat products). The main filamentous fungi associated with fermented foods and their technological functions are shown in **Table 1**.

Table 1. Main filamentous fungi used to manufacture fermented foods and their functional role (adapted and modified from Nout, 2007)

	Species	Food	Functions	Références
Mucoromycota				
<i>Actinomucor</i>	<i>A. elegans</i> , <i>A. taiwanensis</i>	sufu, fu-tu (China)	Texture, aroma	Chou et al., 1988; Han et al., 2001
<i>Amylomyces</i>	<i>A. rouxii</i>	ragi (Indonesia), marcha (India)	Glucose biosynthesis from starch	Tsuyoshi et al., 2005
<i>Mucor</i>	<i>M. circinelloides</i> , <i>M. rouxii</i> , <i>M. indicus</i>	ragi, marcha, tempe (Indonesia), pehtze (China)	Enzymatic transformation, aroma compound production	(Agranoff and Markham, 1997; Pedraza-Reyes and Lopez- Romero, 1991)
	<i>M. lanceolatus</i> , <i>M. fuscus</i> , <i>M. racemosus</i> , <i>M. brunneogriseus</i> , <i>M. spinosus</i>	cheese (France), fermented meat (<i>M. racemosus</i>)	Colour, texture, aroma compound production	Bruna et al., 2001; Hermet et al., 2012
<i>Rhizopus</i>	<i>R. microsporus</i> , <i>R. oligosporus</i> , <i>R.</i> <i>oryzae</i>	koji (Japan), nuruk (Korea), chu (China), marcha, tempe	Enzymatic transformation, texture, vitamins	Ginting and Arcot, 2004; Nout and Kiers, 2005
Ascomycota				
<i>Aspergillus</i>	<i>A. sojae</i> , <i>A. oryzae</i> , <i>A. niger</i>	Soya sauce (Eastern asia)	Glucidolytic enzymes, proteases, other lytic enzymes	Hanya and Nakadai, 2003; Nout and Aidoo, 2002
	<i>A. glaucus</i> , <i>A. repens</i> , <i>A. melleus</i> , <i>A. candidus</i>	katsuobushi (Japan)	Enzymatic transformation, aroma compound production	Campbell-Platt, 1987
<i>Bisifusarium</i> ,	<i>B. domesticum</i> ,	cheese (France,	Colour, texture, aroma	Bachmann et al., 2005; Ropars et

<i>Longinectria</i>	<i>B. allantoides</i> , <i>B. penicilloides</i> , <i>L. lagenoides</i> , <i>L. verticilliforme</i>	Switzerland)	compound production	al., 2012; Savary et al., 2021
<i>Monascus</i>	<i>M. pilosus</i> , <i>M. purpureus</i> , <i>M. ruber</i>	angkak, red rice (China)	Colour, aroma compound production, Secondary metabolites	Akihisa et al., 2005; Martinkova et al., 1996
<i>Neurospora</i>	<i>N. sitophila</i> , <i>N. intermedia</i>	oncom (Indonesia)	Texture, aroma compound production, Enzymatic transformation	Beuchat, 1986
<i>Penicillium</i>	<i>P. glaucum</i>	katsuobushi	Texture, aroma compound production, Enzymatic transformation	Campbell-Platt, 1987
	<i>P. camemberti</i>	Mold-ripened cheese (France)	Colour, texture, aroma compound production	Ropars et al., 2012
	<i>P. nalgiovense</i> , <i>P. camemberti</i>	Fermented meat (Europe)	Colour, aroma compound production, antioxydant activity, Homogenous dehydration of product	Berni and Spotti, 2007
	<i>P. roqueforti</i>	Blue cheeses (Europe)	Colour, texture, aroma compound production	Ropars et al., 2012
<i>Scopulariopsis</i>	<i>S. fusca</i> , <i>S. flava</i> , <i>S. candida</i>	Cheese (France)	Colour, texture, aroma compound production	Ropars et al., 2012
<i>Sphaerosporium</i>	<i>S. equinum</i>	Cheese (France)	Colour, texture, aroma compound production	Ropars et al., 2012
<i>Sporendonema</i>	<i>S. casei</i>	Cheese (France)	Colour, texture, aroma compound production	Ropars et al., 2012

2. Filamentous fungi with undesirable impacts on food quality and safety

As mentioned earlier, molds can also have undesirable impacts on food quality and safety. First, fungi are important contributors to the massive worldwide food losses and waste occurring each year, which represent in total about one-third of the food produced (Cederberg and Sonesson, 2011). Concerning food losses and waste caused by fungi, Pitt and Hocking, (2009) estimated that fungi are responsible for 5-10 % of total food losses and waste. However, surveys specifically dedicated to the quantification of food product loss caused by fungi are scarce in the literature, but earlier studies from Malkki and Ravha (1978) and, more recently, Kim and Xiao (2008), reported that fungi were responsible for 1-3 % of bakery product losses and for 1.9% (fungicide-treated) and 5.1% (untreated) of apple post-harvest losses.

Fungal spoilage can occur at all stages of the food chain, including in the field, post-harvest, during processing and storage by food producers but also during the different food handling steps at the wholesaler, retailer, and consumer's home. In addition, fungi can spoil a very diverse range of foods, from raw materials to processed foods, as they are able to grow over a wide activity water (a_w), pH and temperature range and can use many different substrates, including sugars, organic acids, proteins and lipids. The major fungal groups which are involved in food spoilage can be divided as follows: xerophilic fungi able to grow at $a_w < 0.8$, thermoresistant fungi which can resist heat treatments such as pasteurization, fungi resistant to preservatives (e.g. natamycin, sorbic acid, propionic acid, benzoic acid), fungi able to develop under low O_2 levels and/or in the presence of modified atmosphere and, finally, psychrotolerant or psychrophilic fungi which can grow in refrigerated foods (Rico-Munoz et al., 2019). Given the extent of foods that can be spoiled by filamentous fungi, it is therefore not surprising that from a taxonomical perspective, a wide diversity of fungi are involved in food spoilage. This diversity is illustrated in Figure 1, which shows a heatmap of the different genera involved in the spoilage of the main food categories. This figure was created based on a meta-analysis of the different genera, species and species complexes (83 genera comprising 938 species or species complex) cited in Pitt and Hocking (2009) which is one of the reference books of food mycologists. Based on this meta-analysis, four major conclusions can be drawn. Firstly, many fungal genera and species are involved in food spoilage and a single food category can be spoiled by different fungal genera represented by different species. Secondly, a single genus

can be encountered in different food categories. Thirdly, *Aspergillus*, *Penicillium* and *Fusarium* represent the main genera involved in food spoilage, with *Aspergillus* and *Penicillium* spp. being encountered at relatively high frequency in most, if not all, food categories.

Besides food losses and waste linked to fungal spoilage, growth of certain fungi in certain food commodities, especially agricultural crops, may pose a risk for human and animal health because of their ability to produce mycotoxins. These mycotoxins, which are mainly produced by species of the *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* genera, can have toxic, carcinogenic, and mutagenic reactions in humans at low concentrations after acute, short-term, and/or long-term exposure. Aflatoxins, citrinin, trichothecenes (deoxynivalenol, T-2 and HT-2 toxins), ergot alkaloids, fumonisins, ochratoxin A, patulin and zearalenone are the main mycotoxins of concern from a safety and regulatory point of view (**Table 2**). Several of these fungal secondary metabolites, including aflatoxins, fumonisins, deoxynivalenol, T-2 and HT-2 toxins, zearalenone and ochratoxin A, have been estimated to occur at levels above the Codex and EU limits in 20-25% of cereals and nuts validating FAO estimate, while their occurrence above detectable levels can reach up to 60-80% (Eskola et al., 2020). Noteworthy, mycotoxins present in raw materials can remain in processed foods as they are not very susceptible to technological treatments.

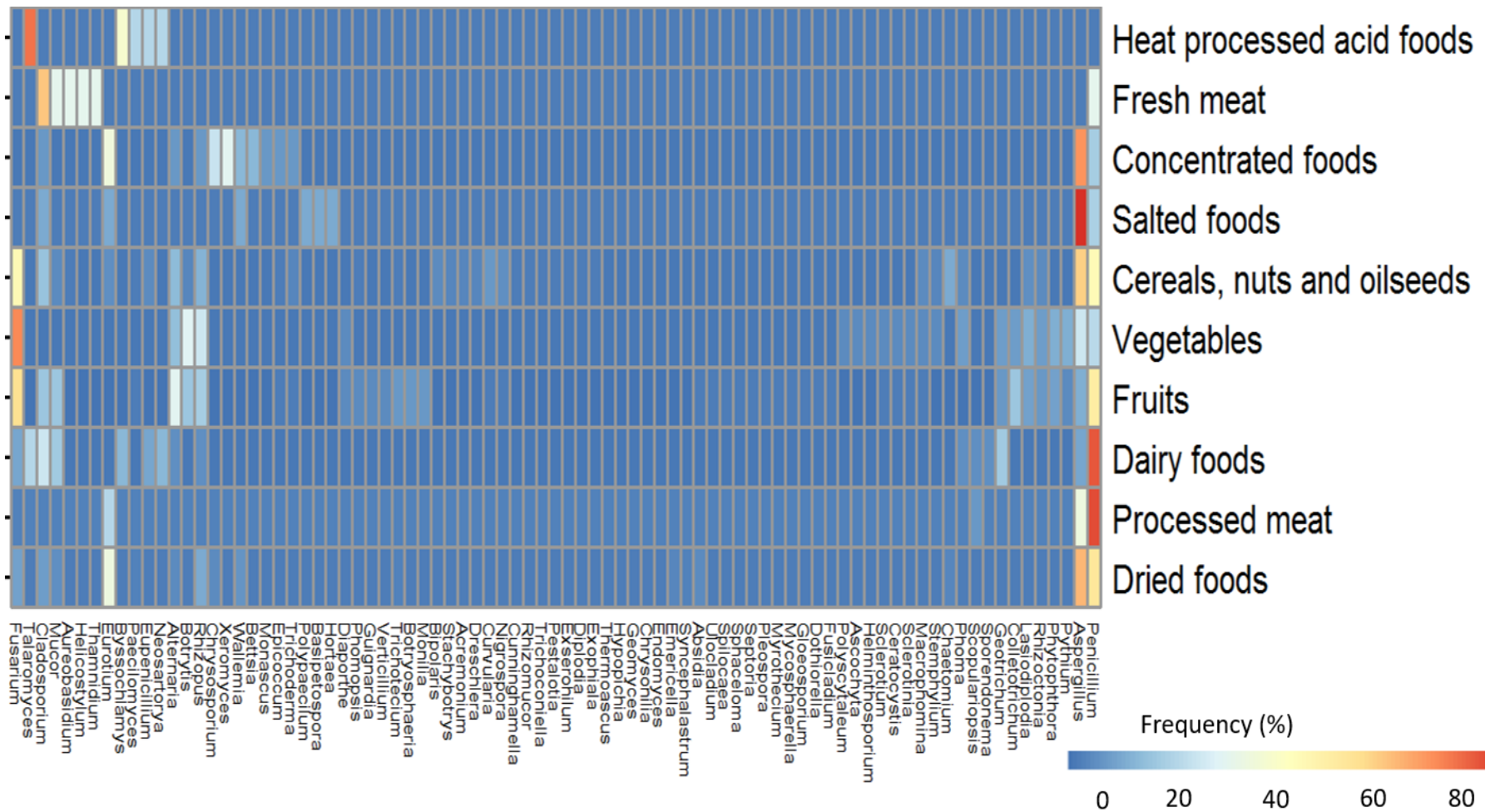


Figure 1. Meta-analysis showing the frequency of main filamentous fungal genera across different food categories cited in Pitt and Hocking (2009) reference book (83 genera comprising 938 species or species complex).

Table 2. Examples of mycotoxins, producing fungi and host plants

Mycotoxin	Producing fungi	Host
Aflatoxins (B1, B2, G1, G2)	<i>Aspergillus flavus</i>	Wheat, barley, corn, peanut
	<i>Aspergillus parasiticus</i>	
Citrinin	<i>Monascus purpureus</i>	Food supplements based on fermented rice
Trichothecenes (deoxynivalenol, T-2, HT-2)	<i>Fusarium</i> spp.	Wheat, barley, oat, corn, rice, rye
Ergot alkaloids	<i>Claviceps purpurea</i>	Rye and other grains
Zearalenone	<i>Fusarium</i> spp.	Corn, wheat, barley, sorgho, rye
Fumonisin (B1, B2, B3)	<i>Fusarium proliferatum</i>	Corn, sorgho, rice
	<i>Fusarium verticilloides</i>	
Ochratoxine A	<i>Aspergillus ochraceus</i>	Corn, grape, barley, soya, coffee and cocoa beans
	<i>Penicillium viridicatum</i>	
	<i>Penicillium cyclopium</i>	
Patulin	<i>Penicillium expansum</i> and other	Pome and stone fruits
	<i>Penicillium</i> spp., <i>Aspergillus</i>	
	<i>clavatus</i> , <i>A. giganteus</i> and <i>A.</i>	
	<i>longivesica</i> , <i>Byssochlamys nivea</i>	

Given the important impact of molds in food, their accurate identification is a key step for managing food safety and quality. Indeed, species-level identification can be a prerequisite for risk assessment with the aim of implementing adequate corrective actions.

III. Identification of fungi

For years, fungal species identification has only been achieved through morphological approaches. Nevertheless, accurate morphological identification of fungi is tedious and challenging; compared with plants and animals, fungi have simple body plans, often with morphologically obscure structures (Lücking et al., 2020). Morphological diagnostic features are usually seen, after cultivation on an agar medium, via examination of their microscopic asexual and/or sexual spore-producing structures as well as the observation of their growth behavior under different abiotic conditions (temperature, aw etc.). Based on these observations, fungal specimens can be identified with the help of dichotomous identification keys such as the widely used *Laboratory Guide to common Penicillium species* (Pitt, 1988) or those available in *Food and Indoor Fungi* (Samson et al., 2019).

Over the last two decades, DNA barcoding has considerably modified our ability to identify species by relying on a single feature: a DNA sequence nucleotide composition. Indeed, DNA barcoding uses a short section of DNA from a specific gene, i.e. a DNA barcode. The DNA barcode is amplified by Polymerase Chain Reaction (PCR) using generic primers (i.e. amplifying the barcode in all species within a group of organisms such as a kingdom) and the resulting amplicons are sequenced to reveal their nucleotide composition. By comparison with a curated reference library of such a DNA barcode, an individual sequence can be used to uniquely identify a specimen. The nucleotide sequence of the selected barcode must be conserved at the species level but variable among species.

In fungi, the universal DNA barcode is the nuclear ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS) (Schoch et al., 2012). The ITS is located between the 18S rRNA and 28S rRNA genes and includes the 5.8S rRNA gene that splits the ITS into two parts: ITS1 and ITS2. The ITS region undergoes a faster rate of evolution than rRNA genes but, fulfilling significant functions during rRNA maturation (Joseph et al., 1999; Liu and Schardl, 1994), they are under selective pressure and remain homogeneous within a species (Liao, 2000).

This intra-species conservation and interspecies variability of its nucleotide composition and its huge representation in reference libraries (e.g. >1,500,000 fungal ITS sequences available in GenBank) make ITS an ideal fungal DNA barcode. Besides ITS, other DNA barcodes may be used in fungi because they offer a better resolution for certain genera, and protein coding genes, such as the β -tubulin encoding gene (Visagie et al., 2014) for *Penicillium* spp. or the elongation factor encoding gene for *Fusarium* spp. (Geiser et al., 2004), are commonly used.

Beyond species identification of fungal isolates, the use of DNA barcodes, when associated with next-generation sequencing (NGS), which allow massive and parallel sequencing, enables culture-independent analyses of fungal communities in complex samples (e.g. food product) and their spatial and temporal dynamics (Grant et al., 2021).

Although DNA barcoding allows for accurate identification, when used routinely, it can be costly, time-consuming and still requires expertise (Chalupová et al., 2014). That is the reason why, techniques such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) which is more rapid, easier to handle but still reliable in identifying filamentous fungi, has emerged in the last decade as a valuable asset in the context of routine identification of fungal isolates in the food industry.

IV. Identification of foodborne molds using MALDI-TOF MS

Rapid bacterial and yeast identification by MALDI-TOF MS has revolutionized microbiology (Patel, 2019) and is now widespread around the world in many different types of laboratories, including food microbiology laboratories. MALDI-TOF MS for mold identification is now well established (Gautier et al., 2014; L'Ollivier and Ranque, 2017) but it took more time for laboratories to adopt this technology in routine practice due to limited database availability and lack of standardized processes (Lau et al., 2019). Identification of molds in general, including foodborne molds, is highly dependent on the sample preparation (Sanguinetti and Posteraro, 2017).

1. Sample preparation

Several protocols are described in the literature, either from liquid culture or from solid medium. When grown on a solid medium, the molds can also be either spotted directly on the target slide used for analysis or can undergo a tube extraction before deposit (Sanguinetti and Posteraro, 2017).

Some initial studies used the direct transfer method (De Carolis et al., 2012). A portion of a grown colony on solid media is directly spotted on the MALDI target slide or suspended in 200 μ L of distilled water before spotting. Each sample is overlaid with 1 μ L of absolute ethanol or formic acid and air-dried at room temperature. One microliter of a saturated solution of α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile/2.5% trifluoroacetic acid is then added and allowed to co-crystallize with the sample at room temperature. This sample preparation has many limitations and can also lead to staff and instrument contamination, especially in case of sporulating fungi. Therefore, studies using direct transfers are outnumbered by studies using a cell lysis /extraction approach, in which an ethanol-formic acid procedure, starting mostly from agar, allows complete protein extraction (Sanguinetti and Posteraro, 2017). The protocol, which involves formic acid and acetonitrile, is safe for the staff and for the instrument and lead to reproducible results. A standardized protocol is described in Quéro *et al.*, 2018. Mold culture (hyphae and/or conidia) are collected from the agar plate using a

moisturized cotton swab, fungal material is then transferred into a microcentrifuge tube containing 900 μ L of 70% ethanol. After homogenization, a first centrifugation step at 14,000 g for 2 min is performed, the supernatant is discarded, the pellet suspended in 40 μ l of 70% formic acid and mixed. Acetonitrile (40 μ L) is then added, before the second step centrifugation at 14,000 g for two minutes. Proteins in suspension in the supernatant are spotted on the target slide and covered with matrix before MALDI-TOF analysis.

This protocol is reproducible and allows spore inactivation. It is easy to perform and does not require specific expertise. The spectra can be different depending on the age of culture and solid media used for extraction (Figure 2). Those parameters should be taken into account for database building.

The sample preparation from liquid culture is not widely used because of its complexity. The strains are grown in liquid medium and incubated at 30°C for 24–48 h (Vidal-Acuña et al., 2018). One milliliter of the liquid fungal culture is placed into a 1.5 ml Eppendorf tube and centrifuged at 15,000 g for two minutes. The supernatant is then discarded and submitted to several washing/centrifugation steps in order to eliminate protein from media. The pellet is resuspended in 300 μ l distilled water and 900 μ l of ethanol is added. After a centrifugation step at 15,000 g for two minutes, the pellet is resuspended in 70% formic acid, incubated for 5–10 min at room temperature, before acetonitrile addition and centrifugation at 15,000 g for one minute. Supernatant is deposited on the target, covered by CHCA matrix and analyzed with MALDI-TOF. This technique is slow, requires specific equipment and mix cultures are not detectable.

Overall, molds identification using MALDI TOF MS needs a standardized protocol which includes an extraction step and preferably from solid culture.

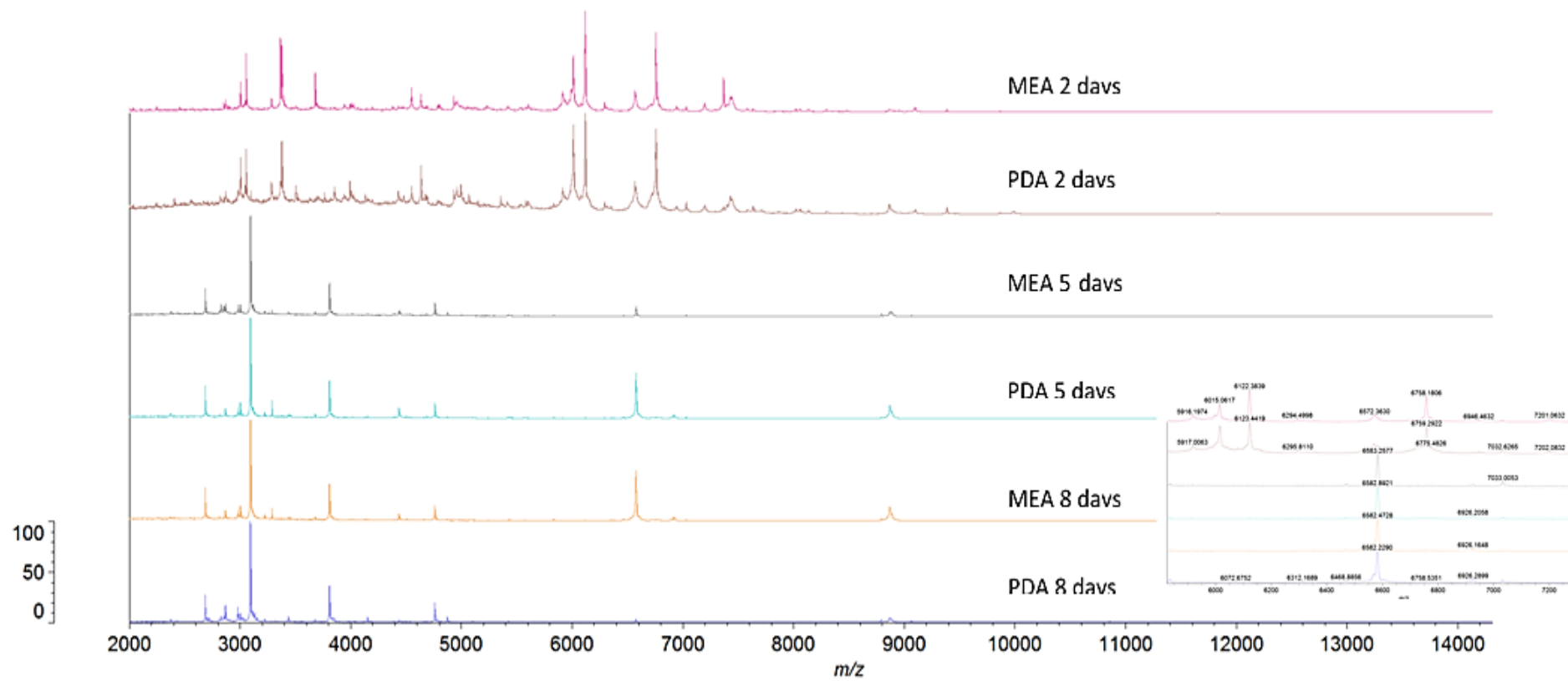


Figure 2. *Aspergillus brasiliensis* ATCC 16404 spectra obtained at 2, 5 and 8 days on both solid media Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA), (Source personnel).

2. Database building and performance of MALDI-TOF for identification of foodborne molds

As previously mentioned, the adoption rate of MALDI-TOF MS for molds was slow, mainly due to poor performance of manufacturer's databases, leading to a heavy dependency on laboratory-developed databases. The species coverage of MALDI reference databases was also the Achilles' heel of MALDI-TOF MS systems when applicable to foodborne molds. These two hurdles have been overcome recently with the recent release of an Food and Drug Administration (FDA)-cleared mold database with reliable sample preparation and performance (Pinheiro et al., 2019; Rychert et al., 2018; Sun et al., 2021) and the work done to expand it to foodborne microorganisms, including molds (Akimowicz and Bucka-Kolendo, 2020; Quéro *et al.*, 2020 and 2018).

a. Database building

To build a robust spectral database, three factors need to be taken into account. The first one is a standardized sample preparation, as explained earlier. The second factor is the microbial variability. To take into account this variability, several strains of the same species should be tested. The recommended number is 10. Those strains should ideally have different geographical origins and should come from different sample types (dairy products, beverages, pastries, etc). This diversity is needed to capture the species characteristics, as different profiles can exist depending on the origin of the strains. The third factor that should be taken into account is the technical variability. As shown in Figure 2, the mold spectra can be different depending on the growth phase. At least two different times of growth should be used to build the database in order to get hyphae and conidia. For example, for rapid growers like *Aspergillus*, spectra should be acquired after two and eight days of growth. Different media and vendors should also be used to represent the diversity of media used in industrial mycology. An example of experimental plan is shown in **Table 3**.

Table 3. Example of an experimental plan for database building for one species. Each strain is tested after 2 days and 8 days of incubation on two different media from two different suppliers.

Strain	Incubation time	Medium	Vendor
Strain 1	2 days	Sabouraud dextrose agar	bioMerieux
Strain 1	8 days	Potato dextrose agar	Becton Dickinson
Strain 2	2 days	Malt extract agar	Oxoid
Strain 2	8 days	Yeast Glucose Chloramphenicol agar	Becton dickinson
Strain 3	2 days	Sabouraud dextrose agar	Becton dickinson
Strain 3	8 days	Potato dextrose agar	bioMerieux
Strain 4	2 days	Malt extract agar	bioMerieux
Strain 4	8 days	Yeast Glucose Chloramphenicol agar	Oxoid

Once spectra are acquired, there are several ways to build databases and estimate performance. The manufacturer bioMérieux developed its own algorithm (Girard et al., 2016) for their MALDI-TOF identification solution, called VITEK® MS. Peak lists are binned and a log base scaling of the peak intensities is applied followed by a L1-normalization. A predictive model is established for each species using the Advanced Spectra Classifier (ASC) algorithm. This procedure provided a specific weighed bin matrix for each species and, for identification, new spectra are compared to the bin weight matrix and the sum of matching bin weights is calculated and considered as an intermediate score. All species-specific scores are then transformed into multiclass probability estimates using a Gaussian-based calibration procedure that takes into account the score distributions within species compared with all other species. To estimate the performance, a five-fold cross-validation is used to assess how accurately it would perform on independent new spectra. The spectral data are randomly split into five subsets. One round of cross-validation involved a learning phase on four subsets and validated the identification performance on the remaining subset. Five rounds of cross-validation are performed by permutation of the subsets. The results are combined across rounds to get an estimation of identification performance. Once the spectral database is built and its performance estimated, it should be challenged with external isolates as described in Quéro *et al.* (2018).

b. Performance of foodborne mold database

Only two studies on foodborne molds and MALDI-TOF MS have been published. The aim of the study of Quéro *et al.* (2018) was to implement a spectral database representative of food spoilage molds. To this end, 6477 spectra were acquired from 618 fungal strains belonging to 136 foodborne species and integrated in a database. The performance of this database was 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. This comprehensive study shows that MALDI-TOF MS can be applied to the identification of foodborne molds with reliable performance when a standardized sample preparation is used. This study also showed that some phylogenetically close species such as *Penicillium camemberti* and *Penicillium commune* could not be separated. By contrast, the species of the *Cladosporium sphaerospermum* complex integrated into the database could be differentiated and correctly identified. Another study in 2020 by the same author (Quéro *et al.*, 2020) showed that the differentiation of species to the complex level can be challenging. The aim of this study was to evaluate the use of MALDI-TOF to identify species of the *Aspergillus* section *Flavi*. Spectra were acquired from 23 *Aspergillus* species and integrated into a database for which cross-validation led to more than 99% of correctly attributed spectra. Even closely related species which cannot be differentiated from a morphological point of view and require a polyphasic analysis, such as *Aspergillus parasiticus* and *Aspergillus sojae*, could be differentiated using MALDI-TOF MS. In the second part of the study, the authors tried to differentiate *Penicillium roqueforti* isolates from three distinct genetic populations. For this study, spectra were acquired from 63 strains and a two-step calibration procedure was applied before database construction. Cross-validation and external validation respectively led to 94% and 95% of spectra, respectively, attributed to the right population. The results suggested very good agreement between spectral and genetic data analysis, demonstrating MALDI-TOF's applicability as a fast and easy alternative to molecular techniques for strain typing of filamentous fungi in combination with the use of alternative algorithms.

Another interesting perspective for the identification of foodborne molds using MALDI-TOF MS is the direct identification from food matrices. Preliminary studies suggest that the identification performance is very variable depending on the food matrix and the fungal species studied. These results also indicate that it is necessary to wait for a significant development of the fungal contaminant to obtain a reliable identification (J. Mounier, personal communication).

Overall, MALDI TOF MS is a promising tool for the identification of foodborne molds now that standardized protocols and robust databases exist. Published studies are scarce and additional work remains to be done to assess the performance and capabilities of such a technique to identify and type the major mold species encountered in food. One challenge will also be to keep the database taxonomy updated as fungal taxonomy is constantly evolving. New species and new strains for species already encountered in the database will also need to be added on a regular basis to ensure that the databases provide the most accurate identification results.

V. Objectifs des travaux de thèse

Au travers de cette synthèse bibliographique, transparaissent les impacts tant négatifs que positifs des organismes fongiques sur les matrices alimentaires (Nout, 2007). Bien que, dans certains cas, ils puissent participer à l'augmentation de la durée de conservation des aliments ou au développement de leurs propriétés organoleptiques, ils peuvent être la source d'altérations. Ces dernières pouvant survenir à toutes les étapes de la chaîne de production et affecter un vaste panel de denrées, elles représentent un enjeu économique substantiel. La détérioration de ces aliments ne constitue pas la seule problématique. En l'occurrence, la potentielle production de mycotoxines par certains organismes présente un risque majeur pour la santé humaine et animale. Comme développé précédemment, la maîtrise de ces contaminants est cruciale pour assurer la sécurité et la qualité des produits et des environnements de production. Cela repose notamment sur la capacité d'identification des organismes fongiques responsables de ces altérations (Croxatto et al., 2012; Pitt and Hocking, 2009).

Durant plusieurs décennies, l'identification reposait essentiellement sur des méthodes phénotypiques, dites «conventionnelles», basées sur l'analyse des caractéristiques morphologiques, biochimiques et culturelles des isolats (Lücking et al., 2020). Ces approches s'avèrent fastidieuses, faiblement discriminantes, peu reproductibles et requièrent une expertise poussée. Des méthodes génotypiques dites « non conventionnelles » se sont développées par la suite. Elles reposent sur le séquençage d'un ou plusieurs gènes d'intérêt taxonomique (code-barres) (Schoch et al., 2012). Si les méthodes moléculaires sont considérées comme une référence pour l'identification des champignons de par leur justesse, leur application au sein d'industries en routine demeure complexe à mettre en œuvre et à automatiser, nécessite une forte expertise et implique des coûts significatifs.

Face à ces problématiques, des méthodes alternatives ont émergé, à l'image de la spectrométrie de masse MALDI-TOF, créant un tournant pour l'identification en routine de microorganismes. Le spectromètre se compose de trois éléments fonctionnels : une source d'ionisation permettant de transférer les ions moléculaires de l'échantillon dans l'analyseur, un analyseur de masse afin de séparer les ions en fonction de leur ratio masse / charge (m/z) et un détecteur (Figure 3).

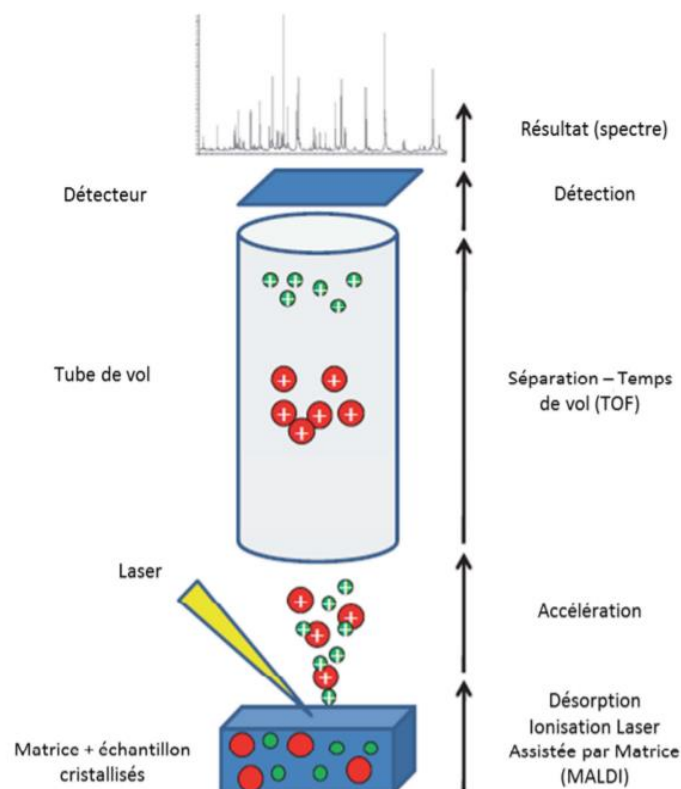


Figure 3. Représentation schématique du mode de fonctionnement d'un spectromètre de masse MALDI-TOF (Quero, 2018 d'après Croxatto et al., 2012)

Cette approche repose sur l'ionisation des molécules d'un échantillon sous l'effet d'un laser, précédée d'une étape de cristallisation. Cette dernière est réalisée grâce à l'ajout d'une matrice ayant une forte capacité d'absorption afin de répercuter l'énergie de la source d'ionisation aux molécules de l'échantillon (Fenselau, 2013). Les molécules ionisées seront alors désorbées et propulsées dans le tube de vol sous vide (analyseur TOF). Lors de cette étape, les ions sont séparés selon leur ratio m/z avant d'être détectés au terme du processus. Les différents rapports m/z et l'abondance des ions correspondants sont alors exposés sous forme de spectre où chaque pic correspond à un biomarqueur et la hauteur des pics est proportionnelle au nombre d'ions de même rapport m/z ayant atteint le détecteur. Cette méthode simple, rapide, peu coûteuse en consommables et précise est applicable à tous types de microorganismes (bactéries, levures, moisissures), (Girard et al., 2016, 2017; Quero, 2018). Elle répond donc à la demande du marché industriel quant à la disposition d'une solution d'identification et même de typage (source-tracking) des champignons d'intérêt alimentaire. Cependant, cet outil reste tributaire des bases de données spectrales.

Un précédent projet de thèse (Thèse CIFRE 2015-2018) a été mené par bioMérieux en collaboration avec le LUBEM avec l'objectif de développer une base de données spectrales comportant des moisissures d'intérêt alimentaire. Les travaux menés par le Dr. Laura Quéro avaient deux axes principaux : le développement de la base de données spectrales et l'étude de la capacité de discrimination du MALDI-TOF dans le cas de complexes d'espèces fongiques ainsi que de populations au sein d'une même espèce.

D'une part, les acquisitions menées ont permis la construction d'une base de données robuste grâce à l'adjonction de 136 espèces distinctes de champignons filamenteux d'intérêt alimentaire (Quéro et al., 2018b). A ce jour, la base de données bioMérieux comprend 221 espèces fongiques. Toutefois, l'extension de la base de données existante doit être poursuivie, notamment au sein de complexes d'espèces, afin de couvrir la très grande diversité de microorganismes fongiques liés aux altérations de denrées alimentaires. De plus, l'ajout de levures d'intérêt alimentaire permettrait de couvrir les besoins des industriels confrontés à diverses problématiques d'altération fongique.

D'autre part, une première preuve de concept concernant la différenciation de complexe d'espèces incluant des espèces cryptiques a été faite dans le cas de la section *Flavi* d'*Aspergillus* mais également lors de l'étude de trois populations de *Penicillium roqueforti* au MALDI-TOF (Quéro et al., 2020). La spectrométrie de masse MALDI-TOF offre donc la possibilité de distinguer des espèces proches, ainsi que différentes populations, là où des approches génotypiques sont habituellement requises. L'application de cette technique à l'identification et au typage des contaminants fongiques dans les produits alimentaires reste à développer et à explorer dans un contexte industriel pour évaluer la capacité de la spectrométrie de masse MALDI-TOF à identifier et typer les isolats responsables de contaminations, ainsi qu'à déterminer leur origine environnementale.

C'est dans ce contexte que s'inscrit ce projet de thèse CIFRE mené par bioMérieux avec la collaboration du LUBEM. La première étude de ce travail doctoral porte sur l'extension de la base de données spectrales VITEK® Mass Spectrometry (MS) Knowledge Base (KB) Fungi grâce à des espèces d'intérêt agro-alimentaire, dont des espèces appartenant à des complexes, puis d'en évaluer les performances.

A travers ce chapitre, nous cherchons à répondre aux questions scientifiques suivantes :

- Est-il possible d'étendre la base de données spectrales levures - moisissures sans dépréciation des capacités d'identification ?
- La spectrométrie de masse MALDI-TOF serait-elle applicable à un plus grand nombre de complexes d'espèces ?
- Le cas échéant, quelle serait sa capacité de discrimination dans le cas de nouveaux complexes, présentant par ailleurs des espèces cryptiques ?

La seconde étude, portant sur la diversité et les voies de contamination de moisissures d'altération de produits de boulangerie en utilisant la spectrométrie de masse MALDI-TOF et des approches moléculaires au sein d'une industrie de BVP, vise à apporter des réponses aux questionnements suivants :

- Quelle est la diversité fongique d'un point de vue qualitatif et quantitatif dans l'environnement d'une usine BVP ?
- Quelles sont les sources environnementales des champignons responsables de l'altération des produits ?
- L'utilisation du MALDI-TOF MS est-elle possible pour étudier la diversité interspécifique et établir la cartographie des flux contaminants au sein d'un environnement industriel ?
- Est-il possible d'étudier la diversité intraspécifique (typage, source-tracking) des organismes fongiques grâce à la spectrométrie de masse MALDI-TOF ?

La recherche d'éléments de réponse à ces questionnements souligne l'importance d'adapter et d'évaluer cette technique pour mieux comprendre et maîtriser les problématiques fongiques. Ainsi, le premier chapitre de ce manuscrit s'intéresse au développement et à l'application de la spectrométrie de masse MALDI-TOF pour l'identification des levures et des champignons filamenteux responsables d'altérations alimentaires. Par ailleurs, l'accent est mis sur l'exploration des potentialités et des limites de la méthode en s'intéressant spécifiquement à son application à des complexes d'espèces présentant, pour certains, des espèces cryptiques. Il pose également les fondations méthodologiques et expérimentales nécessaires à l'ensemble des travaux présentés dans cette thèse.

Chapitre 1 : Développement et application de la spectrométrie de
masse MALDI-TOF pour l'identification des levures et des
champignons filamenteux responsables d'altérations alimentaires

I. Présentation des travaux

L'objectif de cette étude est l'enrichissement d'une base de données spectrales grâce à une sélection d'espèces, majoritairement d'intérêt alimentaire, de levures et de moisissures. Le but est en effet de permettre l'identification rapide des organismes fongiques *via* la spectrométrie de masse MALDI-TOF. Dans cette optique, 380 souches appartenant à 133 espèces et issues de différentes collections, i.e. la collection bioMérieux, l'Université de Bretagne Occidentale Culture Collection (UBOCC), l'Etude En Qualité Alimentaire culture collection (EQUASA), la collection du Westerdijk Fungal Biodiversity Institute, ont été mises à profit afin d'étoffer la base de données. L'étude de la littérature a permis de recenser les espèces d'intérêt, c'est-à-dire les champignons, technologiques et d'altération, présents dans l'industrie (Agarbati et al., 2021; Aponte et al., 2010; Bonatsou et al., 2018; Garnier et al., 2017; Pitt and Hocking, 2009; Quintilla et al., 2018; Solieri and Giudici, 2008). Plusieurs critères ont été considérés lors de la sélection : la fréquence d'apparition dans les aliments, le nombre de produits alimentaires impactés et leur éventuel pouvoir mycotoxinogène. Parmi les espèces sélectionnées, cinq complexes d'espèces distincts ont également été ciblés pour une adjonction en base : *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Fusarium dimerum*, *Mucor circinelloides* et la section *Nigri* d'*Aspergillus*. La finalité était d'évaluer les performances du VITEK® MS sur des espèces proches d'un point de vue taxonomique et phénotypique. Le choix des souches a été fait de telle sorte à privilégier l'ajout de souches types, une caractérisation correcte (identification phénotypique systématique, identification génotypique de 330 des souches pour un total de 380) et le respect des aspects réglementaires. En effet, l'accès et l'utilisation de certaines ressources génétiques / microbiennes peuvent être restreints par certaines réglementations ou législations spécifiques à certaines régions du monde (protocole Nagoya, législations internes, etc. ...).

Afin de construire une base de données la plus robuste possible, il est nécessaire d'inclure une certaine diversité. A cet effet, pour chaque espèce, *a minima* huit spectres doivent être obtenus à partir de deux souches distinctes. Les acquisitions doivent également être effectuées à partir de trois milieux gélosés distincts, l'un est le milieu de référence (Sabouraud Dextrose Agar (SDA)) tandis que les deux autres sont des milieux dit « d'expériences » validés lors d'essais de compatibilité. Les milieux d'expériences employés sont, d'une part, Potato

Dextrose Agar (PDA), Malt Extract Agar (MEA), Yeast Glucose Chloramphenicol agar (YGC) pour les levures et, d'autre part, YGC, MEA, Oxytetracycline Glucose Agar base (OGA) pour les moisissures. Par ailleurs, différents fournisseurs (bioMérieux, Oxoid and BD) sont employés pour représenter plus amplement la diversité des milieux utilisés en mycologie industrielle. De plus, dans le cas des champignons filamenteux, les acquisitions sont réalisées à deux temps de culture différents, afin de prendre en considération les différentes structures fongiques (hyphes et conidies). Ces deux temps doivent être espacés de 6 jours afin d'obtenir à la fois des hyphes et des conidies. Ainsi, pour les moisissures à croissance rapide ou modérée, les spectres sont obtenus après deux et huit jours de croissance, tandis que pour les moisissures à croissance lente, les cultures sont utilisées après huit et quatorze jours. Les levures sont cultivées entre 24 et 48h.

Les extraits fongiques ont ensuite été préparés en suivant les recommandations du fournisseur (bioMérieux, Marcy l'Etoile, France). Les isolats de moisissures ont été soumis à un protocole d'extraction à l'acide formique et à l'acétonitrile grâce au kit VITEK® MS dédié aux champignons filamenteux. Les surnageants obtenus à l'issue de cette phase d'extraction étaient ensuite disposés sur lame puis 1µL de matrice CHCA était ajouté. Dans le cas des levures, les souches étaient directement déposées sur lame à l'aide d'une oese ou d'un stylo préleveur (VITEK PICKME Pen). De l'acide formique était ajouté sur chaque spot à raison de 0,5 µL. Enfin, 1µL de matrice CHCA était déposé sur les spots. Des souches de contrôle qualité, à savoir *Candida glabrata* ATCC MYA-2950 (tests levures) et *Aspergillus brasiliensis* ATCC 16404 (tests moisissures), étaient adjointes chaque jour de test et par lot de réactifs/consommables. Pour chaque lame, le calibrant, *E. coli* ATCC 8739, était disposé sur les spots dédiés. Il est aussi important de noter que les dépôts ont été systématiquement réalisés en duplicata. L'acquisition des spectres a été opérée à l'aide du système VITEK® MS (bioMérieux, Marcy l'Etoile, France) équipé du logiciel d'acquisition Launchpad version 2.9.5.6 sur une plage de masse allant de 2 000 à 20 000 Da.

A partir du jeu de données ainsi constitué, une analyse de performances a été menée. La construction de la base de données repose sur un processus itératif permettant de simuler la performance avec tous les spectres acquis. Une itération constitue une base intermédiaire. En général, une vingtaine d'itérations sont nécessaires à la construction d'une base robuste. Par ailleurs, l'analyse de performance menée a été réalisée grâce à la *cross-validation*. Cette

technique permet d'évaluer avec précision un modèle prédictif appliqué à un jeu de données. Dans les faits, le jeu de données est divisé en 5 parties. L'une des parties constitue le test (prédiction d'identification) tandis que les 4 autres servent à construire l'algorithme (modèle de prédiction). Les calculs sont opérés dans ces conditions puis répétés à 5 reprises en alternant le groupe de spectres représentant la partie test. Les performances obtenues sont finalement moyennées. A l'issue de l'extension de la base de données, une validation externe est faite grâce à des souches non-incluses. Dans les présents travaux, 78 souches ont été choisies pour constituer le jeu de données externes.

Lors de la validation croisée, un taux de spectres correctement attribués était de 96,55 %, avec 3,1 % de spectres non identifiés et 0,35 % de spectres mal identifiés. Un total de 97,12 % des espèces décrites ont présenté une performance comprise entre 80 % et 100 %. Parmi les 139 espèces étudiées, 109 ont obtenu un taux d'identification correct de 100 %, soit 78,42 % des espèces répertoriées. Toutefois, quatre espèces ont obtenu un taux d'identification inférieur à 80 %, à savoir *Aspergillus fischeri* (76,92 %), *A. luchuensis* (77,27 %), *Colletotrichum tropicale* (73,68 %) et *Fusarium verticillioides* (73,53 %). Ces résultats pourraient être liés à des confusions d'identification entre espèces étroitement apparentées dans la base de données. Les espèces au sein des cinq complexes inclus ont obtenu des performances comprises entre 73.68% et 100%. Près de 92% d'entre elles présentent des taux d'identification correcte compris entre 90 et 100%. Dans le cadre de la validation externe, 91.19% des spectres ont été correctement identifiés. Les spectres mal identifiés correspondaient principalement à des espèces proches appartenant au même genre ou au même complexe d'espèces.

Cette étude explore le développement et l'application de la spectrométrie de masse MALDI-TOF pour l'identification des champignons responsables de la détérioration des aliments, en particulier ceux appartenant à des complexes d'espèces. L'objectif principal de cette étude est d'enrichir la base de données VITEK® MS avec des complexes et des espèces fongiques pertinentes pour l'industrie agroalimentaire, afin d'améliorer les capacités et la précision de l'identification des espèces fongiques. L'application de cette technologie dans les contextes de production alimentaire permet non seulement une identification rapide, mais aussi une meilleure gestion des risques associés aux altération et à la production de mycotoxines par certaines espèces fongiques.

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Article

Identification of Food Spoilage Fungi Using MALDI-TOF MS: Spectral Database Development and Application to Species Complex

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Abstract: Fungi, including filamentous fungi and yeasts, are major contributors to global food losses and waste due to their ability to colonize a very large diversity of food raw materials and processed foods throughout the food chain. In addition, numerous fungal species are mycotoxin producers and can also be responsible for opportunistic infections. In recent years, MALDI-TOF MS has emerged as a valuable, rapid and reliable asset for fungal identification in order to ensure food safety and quality. In this context, this study aimed at expanding the VITEK[®] MS database with food-relevant fungal species and evaluate its performance, with a specific emphasis on species differentiation within species complexes. To this end, a total of 380 yeast and mold strains belonging to 51 genera and 133 species were added into the spectral database including species from five species complexes corresponding to *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Fusarium dimerum*, *Mucor circinelloides* complexes and *Aspergillus series nigri*. Database performances were evaluated by cross-validation and external validation using 78 fungal isolates with 96.55% and 90.48% correct identification, respectively. This study also showed the capacity of MALDI-TOF MS to differentiate closely related species within species complexes and further demonstrated the potential of this technique for the routine identification of fungi in an industrial context.



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Keywords: filamentous fungi; yeasts; cryptic species; *Aspergillus*; *Fusarium*; *Mucor*; *Colletotrichum*

1. Introduction

The fungal kingdom is estimated to encompass between 2.2 to 3.8 million species, making it one of the widest groups on Earth [1]. This large group includes diverse eukaryotic microorganisms such as yeasts and filamentous fungi [2]. Those can have either positive or negative impacts on human activities. Indeed, fungi can produce a wide range of pharmaceutical products, enzymes and organic acids [2]. They are also major actors in food and beverage industries due to their ability to modify and improve the organoleptic and nutritional properties of food products from animal and plant origins as well as their ability to increase food shelf-life through fermentation [3–5]. They are involved, for instance, in the manufacturing process of soy sauce, miso, tempeh, mold-ripened cheeses, fermented sausages, bread, kombucha, beer, wine and various spirits.

Conversely, due to their ability to colonize a very large diversity of food raw materials and processed foods along the food chain, fungi are also major contributors to global food losses and waste which represent ~1.3 billion tons each year [6]. As an example, Davies et al. (2021) estimated that fungi were involved in up to 20% of global crop yield losses with at least 125 million tons of the five most cultivated crops lost each year because of fungal growth [6]. Moreover, Pitt and Hocking [5] estimated that fungal spoilage was responsible for 5–10% of food losses and waste. It is also worth mentioning that fungal spoilage leads to substantial financial losses [7,8] and the waste of natural resources (land, water and greenhouse gas emission) and contributes to food insecurity worldwide.

Aspergillus, *Penicillium* and *Fusarium* are the main genera involved in food spoilage. Several species of these genera are mycotoxin producers which represent a major hazard for human and animal health [9,10]. Indeed, of the more than 300 mycotoxins that have been identified so far, 6 of them, namely aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone, are regularly found in food, leading to unpredictable and ongoing food safety problems at a global scale [11]. Furthermore, some species within the genera *Mucor*, *Aspergillus* and *Penicillium*, among others, are also responsible for opportunistic infections in immunocompromised patients [12,13]. In this context, the rapid and accurate identification of fungi at the species level is crucial to ensure food safety and quality [14–16].

Traditionally, fungal identification was performed using phenotypic approaches, i.e., morphological and biochemical characteristics [17,18]. However, those approaches are tedious, time-consuming, may be prone to misidentification and require high expertise [7,8]. Over the last two decades, DNA barcoding, which relies on the sequencing of one or more standardized short DNA regions, has drastically modified the ability to identify fungal species [19]. The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) is, for instance, a highly polymorphic region that is considered the universal barcode marker for fungi [20]. Hence, DNA barcoding is nowadays considered as the gold standard because of its reliability and accuracy but remains at the same time an expensive and tedious method requiring special skills and knowledge to be applicable in routine examination in an industrial setting [19,21].

In recent years, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has emerged as a valuable, rapid, cost-effective and reliable asset for microorganism identification [22,23]. This technique was initially applied to bacterial and yeast species before expanding in recent years to filamentous fungus identification [21,24–26]. MALDI-TOF MS is now commonly used for routine microbial identification in clinical and industrial microbiology laboratories. It relies on the rapid and precise analysis of biomolecules such as proteins, peptides, nucleic acids and lipids yielding a specific spectral signature which can then be identified by comparison to a reference spectral library [7,25,27]. Nevertheless, the lack of available spectra in commercialized databases, particularly for fungi, is still a concern [18,21,28]. Spectral database implementation for food-relevant species is therefore necessary to keep this technique up to date in the face of current challenges including the addition of novel target species or fungal taxonomy updates and changes. Concerning the latter aspect, MALDI-TOF MS has been successfully applied to discriminate species within species complexes [26,29,30]. A species complex is defined as a cluster of closely related species [31] which may include cryptic species [21]. Species within a species complex are difficult to distinguish using traditional phenotypic methods and may require the analysis of several specific genes [32]. Despite their close phylogenetic relatedness, these species may exhibit significant differences in their physiology, metabolic or ecological traits [33] and therefore could have a positive or negative impact on human activities as mentioned above. As an example, Quéro et al. [26] were able to correctly identify species of the *Aspergillus* section *flavi* using MALDI-TOF MS. Noteworthy, this section contains species of both technological and toxigenic interest [34]. The *Aspergillus* section *nigri* is also particularly relevant due to its mycotoxin-producing species and their frequent occurrence in food matrices [33,35]. It is worth noting that this species complex was recently re-examined by Bian et al. [33], and six species were defined within this complex, i.e., *Aspergillus brasiliensis*, *Aspergillus eucalypticola*, *Aspergillus luchuensis*,

Aspergillus niger, *Aspergillus tubingensis* and *Aspergillus vadensis*. As of today, many species complexes remain to be studied using MALDI-TOF MS. Additionally, the rapid and accurate identification of species within species complex using an updated spectral database could facilitate distinguishing species with diverse incidences and boost the prevention and control of fungal spoilage in food.

In this context, the goal of this study was to expand the VITEK[®] MS spectral database with food spoilage fungi and evaluate its performance, with a specific emphasis on species differentiation within species complexes.

2. Materials and Methods

2.1. Fungal Strains

In the present study, the current VITEK[®] MS V3.4 Knowledge Base was expanded through the addition of 380 yeast and mold strains, which corresponded to 51 genera and 133 species, as detailed in Table 1. Spectra were also acquired on strains belonging to species already present in the previous version of the database. Out of these 133 species, 119 corresponded to new species entries, and 14 corresponded to existing species in the database for which additional spectra were acquired on new strains. Moreover, six mold species within species complexes were reworked, without spectra addition, to optimize their identification accuracy. Species selection was based on their agri-food relevance and prevalence, their ability to colonize various food types and their known mycotoxin production. Strains were obtained from several collections, i.e., American Type Culture Collection (ATCC, Manassas, CO, USA), bioMérieux strain collection (Marcy L'etoile, France), EQUASA industrial strain collection (Plouzané, France), Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) and the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). Morphological analysis was performed on all strains to confirm the genus or species. Furthermore, for 330 out of 380 strains, their identification was confirmed using DNA sequencing. One or more genes were sequenced (e.g., ITS region, D1/D2 domain of the 26S rRNA gene, partial β -tubulin gene, partial elongation factor-1 alpha gene, partial actin gene, glyceraldehyde-3-phosphate dehydrogenase gene).

Table 1. The species and strain numbers for each species used to expand the database.

Species	Strain number *
<i>Alternaria brassicicola</i>	UBOCC-A-101046 , UBOCC-A-101047
<i>Alternaria infectoria</i>	EQUASA 414 , EQUASA 415
<i>Arthrographis kalrae</i>	API 2104239 , API 2104240 , API 2104241 , API 2104242
<i>Aspergillus amoenus</i>	EQUASA 1271 , EQUASA 1423 , EQUASA 1492 , EQUASA 1502
<i>Aspergillus cibarius</i>	EQUASA 218 , EQUASA 610 , EQUASA 1328
<i>Aspergillus clavatus</i>	EQUASA 749 , EQUASA 822
<i>Aspergillus creber</i>	EQUASA 1424 , EQUASA 1425 , EQUASA 1491 , EQUASA 1499 , EQUASA 1504
<i>Aspergillus domesticus</i>	UBOCC-A-115038 , UBOCC-A-115040
<i>Aspergillus fischeri</i>	CBS 125813 , CBS 483.65
<i>Aspergillus hiratsukae</i>	EQUASA 854 , EQUASA 1132 , EQUASA 1133 , EQUASA 1435
<i>Aspergillus intermedius</i> **	CBS 108.55 , CBS 523.65 ^{NT} , CBS 117329 , CBS 116.62
<i>Aspergillus jensenii</i>	EQUASA 956 , EQUASA 1262 , EQUASA 1266 , EQUASA 1489
<i>Aspergillus penicilloides</i>	CBS 234.65 , CBS 130294
<i>Aspergillus quadricinctus</i>	CBS 135.52 ^T , CBS 128010
<i>Aspergillus restrictus</i>	UBOCC-A-101080 , CBS 541.65 ^T
<i>Aspergillus sojae</i>	CBS 134.52 , CBS 100928 ^{NT}
<i>Aspergillus tabacinus</i>	EQUASA 1018 , EQUASA 1427 , EQUASA 1488 , EQUASA 1500
<i>Berkeleyomyces basicola</i>	EQUASA 1024 , EQUASA 1088 , EQUASA 1089 , UBOCC-A-101281
<i>Candida carpophila</i>	CBS 5256 ^T , CBS 5257

Table 1. Cont.

Species	Strain number
<i>Candida deformans</i>	EQUASA 711 , EQUASA 1313 , EQUASA 1314 , EQUASA 1320
<i>Candida pseudogleabosa</i>	CBS 6715^T , UBOCC-A-214089
<i>Candida saitoana</i>	CBS 6729 , CBS 940^T
<i>Candida variabilis</i>	EQUASA 816 , EQUASA 817 , EQUASA 1120 , EQUASA 1394
<i>Candida versatilis</i>	CBS 1752^T
<i>Chrysonilia sitophila</i>	UBOCC-A-101030 , UBOCC-A-111120 , UBOCC-A-111121 , UBOCC-A-111122
<i>Chrysosporium keratinophilum</i>	API 2104246 , API 2104247 , API 2104248 , API 2104249
<i>Cladosporium allicinum</i>	EQUASA 453 , EQUASA 1406 , EQUASA 1411
<i>Cladosporium bruhnei</i>	EQUASA 239 , EQUASA 406 , CBS 134.31 , CBS 110024
<i>Cladosporium macrocarpum</i>	EQUASA 70 , EQUASA 91
<i>Colletotrichum karsti</i>	UBOCC-A-116037 , CBS 132134^{NT}
<i>Cunninghamella elegans</i> **	API 2104252
<i>Cystobasidium minutum</i> **	UBOCC-A-214063 , UBOCC-A-214082
<i>Engyodontium album</i>	EQUASA 474 , EQUASA 753 , EQUASA 791 , EQUASA 1066
<i>Eupenicillium lapidosum</i>	EQUASA 1111 , EQUASA 1173 , EQUASA 1181 , EQUASA 1348
<i>Exophiala bergeri</i>	CBS 351.52 , CBS 353.52^T , CBS 102241 , CBS 109786 , CBS 111662 , CBS 119094 , CBS 119099
<i>Exophiala lecanii-corni</i>	CBS 232.39^T , API 2101095 , API 2101096 , API 2101100 , API 2101101 , API 2101102
<i>Exophiala oligosperma</i>	CBS 725.88^T , API 2101105
<i>Exophiala pisciphila</i>	EQUASA 373 , EQUASA 375
<i>Exophiala psychrophila</i>	EQUASA 397 , CBS 191.87^T
<i>Filobasidium magnum</i>	UBOCC-A-214029 , UBOCC-A-214192
<i>Fusarium verticillioides</i> **	CBS 734.97
<i>Geosmithia swiftii</i> **	CBS 116927 , CBS 110774 , CBS 158.67 , CBS 296.48^{NT}
<i>Hannaella luteola</i>	API 9312069 , API 2102042
<i>Helicostylum elegans</i>	EQUASA 401.02 , EQUASA 402.04 , EQUASA 302.01 , EQUASA 408.01
<i>Hortaea werneckii</i>	EQUASA 88 , EQUASA 449 , UBOCC-A-201189 , UBOCC-A-208029
<i>Hyphopichia pseudoburtonii</i>	CBS 5510 , CBS 2455^T , EQUASA 1417
<i>Isomucor fuscus</i>	CBS 254.48^T , UBOCC-A-109168 , UBOCC-A-109169 , CBS 230.29 , UBOCC-A-109167
<i>Kazachstania exigua</i>	CBS 6440 , CBS 135^{NT}
<i>Kazachstania unisporea</i>	CBS 398^T , UBOCC-A-220043 , UBOCC-A-223012 , UBOCC-A-223014
<i>Lachancea kluyveri</i>	CBS 3082^T , UBOCC-A-201045
<i>Linnemannia hyalina</i>	EQUASA 281 , EQUASA 282
<i>Linnemannia zychnae</i>	EQUASA 270 , EQUASA 565
<i>Microdochium nivale</i> **	UBOCC-A-102041 , UBOCC-A-105025 , UBOCC-A-113085 , UBOCC-A-113088
<i>Mucor mucedo</i>	UBOCC-A-109064 , CBS 640.67^{NT} , CBS 887.71
<i>Mucor piriformis</i>	EQUASA 582 , CBS 169.25^{NT}
<i>Nigrospora oryzae</i>	CBS 382.50 , CBS 384.69
<i>Nigrospora phaerica</i>	EQUASA 257 , EQUASA 685
<i>Paraphyton cookei</i>	API 2104256 , API 2104258
<i>Penicillium bifforme</i>	UBOCC-A-110150 , UBOCC-A-112050 , UBOCC-A-112051 , UBOCC-A-112052 , UBOCC-A-112
<i>Penicillium charlesii</i>	CBS 304.48^T
<i>Penicillium corylophilum</i> **	EQUASA 86
<i>Penicillium fellutanum</i>	CBS 229.81^{NT} , UBOCC-A-123037
<i>Penicillium funiculosum</i>	UBOCC-A-101419 , UBOCC-A-112140
<i>Penicillium glandicola</i>	CBS 498.75^{ET} , UBOCC-A-110041
<i>Penicillium griseofulvum</i>	UBOCC-A-101424 , UBOCC-A-109220
<i>Penicillium islandicum</i>	UBOCC-A-101425 , CBS 394.50 , CBS 165.81
<i>Penicillium janczewskii</i>	UBOCC-A-111140 , UBOCC-A-113046
<i>Penicillium janthinellum</i>	UBOCC-A-101427 , UBOCC-A-111189
<i>Penicillium macrosporum</i> **	CBS 350.72 , CBS 118884 , CBS 130.89 , CBS 317.63^T
<i>Penicillium olsonii</i>	UBOCC-A-117001 , UBOCC-A-117002 , UBOCC-A-118059 , UBOCC-A-118158
<i>Penicillium purpurogenum</i>	CBS 128132 , CBS 128133 , CBS 184.27
<i>Penicillium rubens</i>	EQUASA 869 , EQUASA 954 , EQUASA 955 , EQUASA 1265 , EQUASA 1268 , EQUASA 1490 , EQUASA 1509
<i>Penicillium rugulosum</i>	EQUASA 936 , EQUASA 1506 , UBOCC-A-111174 , UBOCC-A-111181 , UBOCC-A-111190
<i>Penicillium ubiquetum</i>	EQUASA 125 , EQUASA 129

Table 1. Cont.

Species	Strain number
<i>Phoma pinodella</i>	<u>UBOCC-A-116004</u> , <u>CBS 531.66</u> , CBS 133.92, CBS 123522, <u>CBS 403.65</u>
<i>Pichia occidentalis</i>	<u>CBS 10322</u> , <u>CBS 6399</u>
<i>Rhinocladiella similis</i>	<u>EQUASA 529</u> , <u>EQUASA 942</u>
<i>Rhizomucor pusillus</i>	<u>UBOCC-A-101365</u> , <u>UBOCC-A-111202</u> , <u>API 2104260</u> , <u>API 2104264</u>
<i>Saccharomyces cariocanus</i>	<u>UBOCC-A-220015</u> , <u>UBOCC-A-220031</u> , <u>UBOCC-A-220045</u>
<i>Saccharomyces uvarum</i>	<u>CBS 377</u> , <u>CBS 395</u> [†] , <u>UBOCC-A-201049</u>
<i>Saccharomycopsis fibuligera</i>	<u>UBOCC-A-212006</u> , <u>UBOCC-A-212010</u> , <u>EQUASA 1082</u> , <u>EQUASA 823</u>
<i>Schizophyllum commune</i>	<u>API 2104268</u> , <u>API 2104269</u>
<i>Scopulariopsis asperula</i> **	<u>UBOCC-A-101272</u> , <u>UBOCC-A-108119</u> , <u>UBOCC-A-110145</u> , <u>UBOCC-A-113016</u>
<i>Scopulariopsis candida</i>	<u>UBOCC-A-108117</u> , <u>UBOCC-A-110144</u> , <u>UBOCC-A-113025</u>
<i>Scopulariopsis flava</i>	<u>UBOCC-A-108118</u> , <u>UBOCC-A-113028</u>
<i>Sporobolomyces roseus</i>	<u>UBOCC-A-214093</u> , <u>UBOCC-A-214118</u> , <u>CBS 486</u> ^{LT}
<i>Stachybotrys chartarum</i>	API 2104273, API 2104274, API 2104275, API 2104276
<i>Starmerella etchellsii</i>	<u>CBS 1750</u> [†] , <u>CBS 1751</u>
<i>Syncephalastrum racemosum</i>	<u>API 2104281</u> , <u>API 2104282</u>
<i>Thamnidium elegans</i>	<u>CBS 642.69</u> , <u>CBS 341.55</u>
<i>Trichoderma atroviride</i>	<u>UBOCC-A-101288</u>
<i>Trichoderma harzianum</i> **	<u>UBOCC-A-118023</u> , <u>CBS 226.95</u> ^{NT} , <u>UBOCC-A-117301</u>
<i>Trichoderma viride</i> **	<u>UBOCC-A-101292</u>
<i>Trigonopsis californica</i>	<u>CBS 5383</u> , <u>CBS 5654</u>
<i>Verticillium albo-atrum</i>	<u>EQUASA 1143</u> , <u>UBOCC-A-101307</u>
<i>Verticillium dahliae</i>	<u>UBOCC-A-101312</u> , <u>UBOCC-A-101313</u> , <u>UBOCC-A-101314</u> , <u>UBOCC-A-101317</u>
<i>Verticillium nonalfalfae</i>	<u>EQUASA 203</u> , <u>EQUASA 589</u> , <u>EQUASA 590</u> , <u>UBOCC-A-112135</u>
<i>Wallemia muriae</i>	<u>CBS 116628</u> ^{NT} , CBS 110619, CBS 110624
<i>Xeromyces bisporus</i>	<u>CBS 469.59</u> , <u>CBS 347.94</u> , <u>CBS 236.71</u>
<i>Zygorhynchus florentina</i>	<u>CBS 748</u> , <u>CBS 6703</u> , <u>CBS 6761</u>
<i>Zygorhynchus mrakii</i>	<u>UBOCC-A-220020</u> , <u>UBOCC-A-220022</u> , <u>UBOCC-A-220023</u> , <u>UBOCC-A-220024</u> , <u>UBOCC-A-220025</u>
<i>Aspergillus series Nigri</i>	
<i>Aspergillus brasiliensis</i> **	<u>ATCC 16404</u>
<i>Aspergillus luchuensis</i> (ex <i>Aspergillus coreanus</i>)	<u>CBS 119384</u> , <u>EQUASA 756</u> , <u>EQUASA 1073</u> , <u>EQUASA 1170</u>
<i>Aspergillus eucalypticola</i>	<u>CBS 122712</u> ^{HT}
<i>Aspergillus niger</i> (ex <i>Aspergillus foetidus</i>) ***	<u>CBS 114.49</u> , <u>CBS 121.28</u> ^{NT}
<i>Aspergillus niger</i> (ex <i>Aspergillus lacticoffeatus</i>) ***	<u>CBS 101884</u> , <u>CBS 101885</u> , <u>CBS 101886</u>
<i>Aspergillus luchuensis</i> (ex <i>Aspergillus piperis</i>)	<u>CBS 112811</u> , <u>CBS 113.52</u> , <u>CBS 113.33</u>
<i>Aspergillus niger</i> ***	API 1006067, API 1006068, API 1105141, API 1212008, <u>UBOCC-A-101072</u> , <u>UBOCC-A-101075</u> , <u>CBS 554.65</u> , <u>UBOCC-A-112064</u> , <u>UBOCC-A-112068</u> , <u>UBOCC-A-112080</u> , <u>UBOCC-A-112082</u>
<i>Aspergillus tubingensis</i> **	<u>CBS 115656</u> ^{HT} , <u>CBS 115657</u> , CBS 132411, CBS 563.65, <u>CBS 115574</u>
<i>Aspergillus vadensis</i>	<u>CBS 113226</u> , <u>CBS 113365</u>
<i>Bisifusarium dimerum</i> complex	
<i>Bisifusarium allantoides</i>	<u>UBOCC-A-120035</u> , <u>UBOCC-A-120036</u> , <u>UBOCC-A-120037</u>
<i>Bisifusarium biseptatum</i>	<u>CBS 110138</u> , <u>CBS 110306</u> , <u>CBS 110144</u>
<i>Bisifusarium delphinoides</i>	<u>CBS 115321</u> , <u>CBS 101047</u>
<i>Bisifusarium dimerum</i> ***	SA132479, SA131363, SA131166
<i>Bisifusarium domesticum</i> ***	<u>UBOCC-A-109095</u> , <u>UBOCC-A-113010</u> , <u>CBS 244.82</u>
<i>Bisifusarium lunatum</i>	<u>UBOCC-A-118038</u> , <u>CBS 632.76</u> ^{NT}
<i>Bisifusarium nectrioides</i>	<u>CBS 176.31</u> ^{LT}
<i>Bisifusarium penicillioides</i>	<u>UBOCC-A-120021</u> [†] , <u>UBOCC-A-120034</u> , <u>UBOCC-A-120054</u>
<i>Bisifusarium penzigii</i>	<u>CBS 317.34</u> ^{HT} , <u>EQUASA 1440</u> , <u>EQUASA 1441</u> , <u>EQUASA 1442</u>
<i>Colletotrichum acutatum</i> complex	
<i>Colletotrichum acutatum</i>	<u>UBOCC-A-117265</u> , <u>CBS 126505</u> , <u>CBS 129952</u> , <u>CBS 129914</u>

Table 1. Cont.

Species	Strain number
<i>Colletotrichum fioriniae</i>	<u>UBOCC-A-116032</u> , <u>UBOCC-A-117425</u> , <u>CBS 128517</u> ^T , <u>UBOCC-A-116034</u> , <u>UBOCC-A-116033</u> , <u>UBOCC-A-121023</u> , <u>UBOCC-A-103034</u>
<i>Colletotrichum godetiae</i>	<u>CBS 133.44</u> ^T , <u>UBOCC-A-121017</u> , <u>UBOCC-A-121021</u> , <u>UBOCC-A-115012</u>
<i>Colletotrichum lupini</i>	<u>UBOCC-A-118145</u> , <u>UBOCC-A-118146</u> , <u>UBOCC-A-118147</u> , <u>CBS 109221</u> ^{HT} , <u>CBS 109225</u>
<i>Colletotrichum nymphaeae</i>	<u>UBOCC-A-117287</u> , <u>CBS 515.78</u> ^{ET}
<i>Colletotrichum gloeosporioides</i> complex	
<i>Colletotrichum fructicola</i>	<u>UBOCC-A-118064</u> , <u>UBOCC-A-118065</u>
<i>Colletotrichum gloeosporioides</i>	<u>UBOCC-A-116039</u> , <u>UBOCC-A-116038</u> , <u>UBOCC-A-116036</u>
<i>Colletotrichum musae</i>	<u>UBOCC-A-121003</u> , <u>UBOCC-A-121004</u>
<i>Colletotrichum siamense</i>	<u>UBOCC-A-121006</u> , <u>UBOCC-A-121020</u> , <u>CBS 125379</u>
<i>Colletotrichum tropicale</i>	<u>UBOCC-A-121005</u> , <u>CBS 124949</u> ^{HT} , <u>CBS 125389</u>
<i>Mucor circinelloides</i> complex	
<i>Mucor bainieri</i>	<u>CBS 293.63</u> ^{IT}
<i>Mucor circinelloides</i> ***	<u>UBOCC-A-109182</u> , <u>UBOCC-A-109183</u> , <u>CBS 195.68</u> , <u>UBOCC-A-109192</u>
<i>Mucor ctenidius</i>	<u>CBS 433.87</u> , <u>CBS 696.76</u> , <u>CBS 293.66</u>
<i>Mucor griseocyanus</i>	<u>CBS 116.08</u> , <u>CBS 223.56</u>
<i>Mucor janssenii</i>	<u>CBS 232.29</u> , <u>CBS 185.68</u> , <u>CBS 205.68</u>
<i>Mucor lusitanicus</i>	<u>CBS 633.65</u> , <u>CBS 851.71</u> , <u>CBS 242.33</u>
<i>Mucor ramosissimus</i>	<u>CBS 135.65</u> ^{NT}
<i>Mucor variicolumellatus</i>	<u>CBS 236.35</u> ^{HT}
<i>Mucor velutinosus</i> **	<u>EQUASA 1551</u>
<i>Thamnidium anomalum</i>	<u>CBS 697.76</u> , <u>CBS 243.57</u> ^T

* API, bioMérieux culture collection strains; ATCC, American Type Culture Collection; CBS, Westerdijk Fungal Biodiversity Institute culture collection; EQUASA, Etude En Qualité Alimentaire culture collection; UBOCC, Université de Bretagne Occidentale Culture Collection; SA, external site development collection. ** Species already present in the database for which additional strains were integrated. *** Species from species complexes already present in the database and reviewed for the VITEK® MS Knowledge Base Version 3.4. An underlined strain number indicates that strain identification was also confirmed by DNA sequencing. ^T Type strain ^{HT} Holotype ^{ET} Epitype ^{LT} Lectotype ^{NT} Neotype ^{IT} Isotype.

An external validation was performed to challenge the extended database using 78 strains (Table 2). These strains were obtained from the UBOCC, EQUASA (Plouzané, France) and LUBEM (Plouzané, France) collections. All strains were identified by the DNA sequencing of one or more regions. The list of chosen strains comprised a total of 61 species, including 47 mold species and 14 yeast species. Additionally, 58 species were represented in the extended version of the database, among which 21 represented newly added species, and 3 were extended with additional spectra. The remaining three species were absent from the spectral database.

Table 2. Species and strain numbers used for external database validation.

Species	Strain number
<i>Alternaria brassicicola</i>	M1-0046
<i>Arthrographis kalrae</i> *	API 2104244
<i>Aspergillus amoenus</i> *	EQUASA 1261
<i>Aspergillus cibarius</i> *	EQUASA 610
<i>Aspergillus creber</i> *	EQUASA 491, EQUASA 1169
<i>Aspergillus hiratsukae</i> *	EQUASA 1436
<i>Aspergillus jensenii</i> *	EQUASA 2677
<i>Aspergillus tubingensis</i> *	M1-0085
<i>Aureobasidium pullulans</i>	L1-0011
<i>Bisfusarium bisepatum / penzigii</i> *	EQUASA 1442
<i>Botrytis cinerea</i>	M1-0123, M2-0036
<i>Candida famata</i>	L1-0009
<i>Candida guilliermondii</i>	L1-0006
<i>Candida hellenica</i>	L1-0022
<i>Candida pulcherrima</i>	L2-0005

Table 2. Cont.

Species	Strain number
<i>Chrysonilia sitophila</i> *	UBOCC-A-111123, UBOCC-A-111124
<i>Chrysosporium keratinophilum</i> *	API 2104251
<i>Cladosporium cladosporioides</i> complex	M1-0045, M1-0126, M2-0041
<i>Cladosporium snafimbriatum</i> **	M2-0010
<i>Cladosporium oxysporum</i>	M1-0011
<i>Cladosporium ramotenellum</i>	M1-0014
<i>Colletotrichum lupini</i> *	UBOCC-A-118080, UBOCC-A-118081
<i>Engyodontium album</i> *	EQUASA 473
<i>Eupenicillium lapidosum</i> *	EQUASA 1446
<i>Exophiala dermatitidis</i>	L1-0023
<i>Fusarium proliferatum</i>	M1-0077, M1-0116
<i>Fusarium sambucinum</i>	M1-0137
<i>Fusarium solani</i> complex	M1-0061
<i>Geotrichum candidum</i>	M1-0054
<i>Hortaea werneckii</i> *	EQUASA 680, EQUASA 1364, EQUASA 1365
<i>Kloeckera apiculata</i>	L1-0015
<i>Microdochium nivale</i> *	UBOCC-A-102027
<i>Mucor brunneogriseus</i> **	M1-0063
<i>Mucor circinelloides</i>	M1-0152
<i>Mucor plumbeus</i>	M1-0139, M1-0153
<i>Mucor piriformis</i> *	M2-0003
<i>Papiliotrema laurentii</i>	L1-0007
<i>Penicillium adametzioides</i>	M1-0020
<i>Penicillium aurantiogriseum</i>	M1-0150
<i>Penicillium aurantiogriseum</i> var. <i>polonicum</i>	M1-0001
<i>Penicillium bifforme</i> *	UBOCC-A-112057, UBOCC-A-112058, UBOCC-A-112059
<i>Penicillium brevicompactum</i>	M1-0025
<i>Penicillium citrinum</i>	M1-0049
<i>Penicillium crustosum</i>	M1-0134, M1-0149
<i>Penicillium digitatum</i>	M2-0006
<i>Penicillium expansum</i>	M1-0079
<i>Penicillium glabrum</i>	M2-0033
<i>Penicillium italicum</i>	M1-0144
<i>Penicillium olsonii</i> *	UBOCC-A-118177, UBOCC-A-118178
<i>Penicillium paneum</i>	M1-0109
<i>Penicillium rubens</i> *	EQUASA 1459, EQUASA 1448
<i>Penicillium solitum</i>	M1-0047
<i>Phoma glomerata</i>	M1-0108, CBS 318.90
<i>Rhizopus stolonifer</i>	M1-0098
<i>Rhodotorula babjevae</i> **	L1-0002
<i>Rhodotorula mucilaginosa</i>	L1-0018
<i>Sporobolomyces roseus</i> *	UBOCC-A-208018
<i>Trichoderma harzianum</i> *	M1-0140
<i>Trichoderma viride/ghanense</i> *	M1-0081
<i>Verticillium nonalfalfae</i> *	EQUASA 526
<i>Zygorhizopus mrakii</i> *	UBOCC-A-220032, UBOCC-A-220040

* Species added to the spectral database in the present study.** Species not included in the database.

2.2. Strains Inoculation and Cultivation

First, cryopreserved strains were pre-cultured on Sabouraud Dextrose Agar (SDA, bioMérieux, Marcy l'Etoile, France) at 25 °C typically for 2 to 6 d to assess viability and purity. Prior to spectrum acquisition, yeasts were grown at 25 °C for 2 d before spectra acquisition on four different media, i.e., SDA, Malt Extract Agar (MEA), Yeast Glucose Chloramphenicol agar (YGC) and Oxytetracycline Glucose Agar base (OGA). SDA, MEA and YGC were obtained from three different suppliers, i.e., bioMérieux (Marcy l'Etoile, France), Becton Dickinson (BD, Le Pont de Claix, France) and Oxoid—Thermo Fischer

Scientific (Dardilly, France), while OGA was obtained from Condalab (Torrejón d'Aedoz, Spain). Molds were cultivated at 25 °C for 2 and 8 d before spectra acquisition with the exception of *Aspergillus restrictus*, *Cladosporium allicinum*, *Penicillium funiculosum*, *Penicillium islandicum* and *Xeromyces bisporus* which were incubated for 8 and 14 d due to their slow growth. Four different media, SDA, MEA, YGC and Potato Dextrose Agar (PDA), from 3 suppliers (bioMérieux, Oxoid and BD) were used for cultivation. The extreme xerophile, *X. bisporus*, was grown in an inhouse agar medium (275 g/L glucose, 275 g/L fructose, g/L malt extract, 2.5 g/L yeast extract, 10 g/L agar, a_w 0.84) as described previously [36].

2.3. Mold Sample Preparation

After cultivation for 2 and 8 d as mentioned above, mold isolates were subjected to an extraction protocol using the VITEK[®] MS mold kit (bioMérieux, Marcy l'Etoile, France). Briefly, the mycelium and/or the conidia were sampled on the agar plate surface (approximately 1 cm²) using a sterile cotton swab moisturized with API Suspension Medium (bioMérieux, Marcy l'Etoile, France) [18]. The sample was then immersed into a microcentrifuge tube filled with 900 µL of 70% ethanol (bioMérieux, Marcy l'Etoile, France). After vortexing for 5 s and centrifugation for 2 min at 14,000 × *g*, the supernatant was discarded, and the pellet was resuspended into 40 µL of 70% formic acid (bioMérieux, Marcy l'Etoile, France). After vortexing for 5 s, 40 µL of acetonitrile was added and vortexed again for 5 s. Finally, a 2 min centrifugation was carried out at 14,000 × *g*, and the supernatant was kept for spectra acquisition.

2.4. Spectra Acquisition

For spectra acquisition, two distinct protocols were applied for yeast and mold isolates. For yeast isolates, one colony was randomly collected using a loop or the VITEK[®] PICKME[™] (bioMérieux, Marcy l'Etoile, France) and then smeared in duplicate on a target slide (bioMérieux, Marcy l'Etoile, France). Then, 1 µL of 70% formic acid (bioMérieux, Marcy l'Etoile, France) was added directly to each spot and left to dry. For mold isolates, 1 µL of the previously obtained supernatant was transferred in duplicate on the target slide and allowed to dry. Then, for both yeasts and molds, 1 µL of α-cyanohydroxycinnamic acid matrix solution (CHCA, bioMérieux, Marcy l'Etoile, France) was applied, and the spots were left to dry before MALDI-TOF MS analysis.

Spectra acquisition was performed using the VITEK[®] MS system (bioMérieux, Marcy l'Etoile, France) equipped with the Launchpad version 2.9.5.6 acquisition software. As described by Girard et al. [28], spectra were acquired in linear positive extraction mode in a mass range from 2000 to 20,000 Da using the “Auto-Quality” option. Each spectrum was generated by the accumulation of 500 laser shots, 100 profiles being acquired from each spot with five shots per profile. Calibration was externally made using fresh cells of *Escherichia coli* ATCC 8739. Two quality control strains, *A. brasiliensis* ATCC 16404 for molds and *Candida glabrata* MYA—3950 for yeasts, were also included for each reagent kit and on each day of spectra acquisition. The Launchpad acquisition software automatically processed raw spectra through smoothing and peak detection procedures [28].

2.5. Spectra Quality Control Procedure

Raw spectra were individually controlled for peak resolution, the signal-to-noise ratio and absolute signal intensity. Spectra used to develop the spectral database present typically between 80 and 200 peaks. Good-quality spectra were subsequently transformed into peak lists containing *m/z* values and corresponding intensities [28]. A single linkage agglomerative clustering algorithm was used to generate dendrograms for each species, comparative dendrograms with closely related species and dendrograms involving spectra already included in the database when needed. Dendrograms were then analyzed to detect any doubtful strains and confirm dataset consistency. The acceptance criteria were a minimum of 50% similarity and 50 peaks in common between individual spectra for a

given mold species, while a minimum similarity of 65% and 50 peaks in common were used for yeast species.

2.6. Non-Supervised Analysis of Spectra from Species within Species Complexes

In the case of species complexes in the database, a non-supervised approach was employed as the first step to assess the discriminatory ability of MALDI-TOF MS. The t-Stochastic Neighbor Embedding (SNE) method was used to visualize the distance between spectra in each species complex using Plotly.js V 2.27.0 [37,38]. This non-linear projection technique enables the visualization of high-dimensional data in a lower dimension, typically a two- or three-dimensional map. The high-dimensional data are converted into a matrix of pairwise similarities followed by the application of t-SNE and visualized in a scatterplot [38]. This dimensionality reduction method aims to preserve as much of the significant structure of the initial data while balancing attention between local and global aspects, thereby reducing the tendency for data points to crowd densely in the center of the map [39].

2.7. Development of Spectral Database

As previously described by Girard et al. [28], each peak from the peak list was assigned to one of the 1300 bins within the mass range of 3000 to 17,000 Da [40]. Then, a log base scaling of the peak intensities was applied followed by an L1-normalization. For each species, a predictive model was established using the Advanced Spectra Classifier (ASC) algorithm developed by bioMérieux to obtain a specific weight bin matrix. To provide an identification, the new spectra were compared to the bin weight matrix, and the sum of matching bin weights was calculated and then considered as an intermediate score [28]. The resulting specific scores were transformed into multiclass probability estimates using a Gaussian calibration procedure. A decision algorithm was used to retain only significant matches. When only one species was retained, the result was considered as a 'single choice'. A 'low discrimination' result was obtained when more than one species was proposed, while a 'no identification' result was obtained either when no significant matches were found or if more than four different species were retained.

2.8. Evaluation of Identification Performance by Cross-Validation

A 5-fold cross-validation was used to optimize the VITEK[®] MS Knowledge Base and to assess how accurately it would perform on independent new spectra. This process was based on the partitioning of the spectral data into five complementary subsets. As described by Girard et al. [28], one round of cross-validation involved a learning phase on four subsets and the validation of the predictive model on the remaining subset. Five rounds of cross-validation were performed by the permutation of the subsets. The estimated identification performance was obtained by combining the results of each round. A 'correct identification' was attributed when the same identification results were obtained between the cross-validation and reference identification. A 'low discrimination' result was considered correct if the expected identification was included among the matches. A 'misidentification' was considered as a discordant identification between the cross-validation and reference identification. A 'no identification' result could also occur implying that the spectrum was considered not identified in this case.

2.9. Evaluation of Identification Performance by External Validation

The spectral database was challenged using an external dataset of 78 strains. For cultivation, a medium among those cited above was randomly chosen for each strain. Yeasts were incubated for 2 d before spectra acquisition, while mold isolates were analyzed at two randomly selected incubation times ranging from 2 to 8 d. Positive and negative controls were made using the quality control strains and reagents only, respectively. Spectra acquisition was performed in duplicate as described above. The obtained spectra were compared to the constructed spectral database to evaluate the percentage of correct identifi-

cation for the species claimed in the database and the absence of identification for those not included in the database.

3. Results

3.1. Performance Estimation by Cross-Validation and Database Validation

3.1.1. Performance Evaluation by Cross-Validation

The database performance for each species was estimated using cross-validation. Overall, 96.55% of the spectra from the VITEK MS fungal knowledge base were correctly identified to the species level, 3.1% were not identified and 0.35% were erroneously identified (discordant status).

Among the 139 species added to the spectral database, 109 yielded an overall correct identification rate of 100% after cross-validation (Table 3). These species also yielded 100% of spectra assigned as a single choice except for six of them, namely *Aspergillus amoenus*, *Aspergillus tabacinus*, *Candida variabilis*, *Penicillium bifforme*, *P. funiculosum* and *Penicillium rubens*, which yielded between 2.38% and 12.28% spectra with low discrimination for *A. tabacinus* and *P. bifforme*, respectively. Among the remaining species, an overall correct identification percentage above 90% was achieved for 21 species ranging from 90.91% to 98.57% for *Aspergillus jensenii* and *Penicillium macrosporum*, respectively, while for 5 species (i.e., *Aspergillus creber*, *A. restrictus*, *Cladosporium macrocarpum*, *Colletotrichum siamense*, *Hannaella luteola*), the percentage of spectra correctly identified was between 80% and 90%. Finally, the spectra of four species had levels of correct identification below 80%, i.e., *Aspergillus fischeri* (76.92%), *A. luchuensis* (77.27%), *Colletotrichum tropicale* (73.68%) and *Fusarium verticillioides* (73.53%). *A. fischeri* and *A. luchuensis* had a low percentage of discordant and low discriminant spectra. For *A. fischeri*, 15.38% spectra were identified as a species from the same genus, i.e., *A. coreanus*, while 7.69% of spectra yielded low discrimination results with *A. coreanus* as well. Concerning *A. luchuensis*, 18.18% of spectra were only identified as belonging to the *Aspergillus* series *Nigri*. Furthermore, 26.32% and 25% of spectra from *C. tropicale* and *F. verticillioides* were not identified, respectively.

Table 3. The performance evaluation of the database by cross-validation.

Species	Overall correct (%) ^(a)	Single choice (%)	Low discrimination (%)	No identification (%)	Discordant (%)
<i>Alternaria brassicicola</i>	100	100	0	0	0
<i>Alternaria infectoria</i>	100	100	0	0	0
<i>Arthrographis kalrae</i>	100	100	0	0	0
<i>Aspergillus amoenus</i>	100	89.47	10.53	0	0
<i>Aspergillus cibarius</i>	100	100	0	0	0
<i>Aspergillus clavatus</i>	98.53	98.53	0	1.47	0
<i>Aspergillus creber</i>	89.19	81.08	8.11	10.81	0
<i>Aspergillus domesticus</i>	100	100	0	0	0
<i>Aspergillus fischeri</i>	76.92	69.23	7.69	7.69	15.38
<i>Aspergillus hiratsukae</i>	98.36	98.36	0	1.64	0
<i>Aspergillus intermedius</i> *	100	100	0	0	0
<i>Aspergillus jensenii</i>	90.91	86.36	4.55	4.55	4.55
<i>Aspergillus penicilloides</i>	100	100	0	0	0
<i>Aspergillus quadricinctus</i>	92.86	92.86	0	7.14	0
<i>Aspergillus restrictus</i>	87.5	87.5	0	3.13	9.38
<i>Aspergillus sojae</i>	100	100	0	0	0
<i>Aspergillus tabacinus</i>	100	97.62	2.38	0	0
<i>Berkeleyomyces basicola</i>	100	100	0	0	0
<i>Candida carpophila</i>	91.67	91.67	0	8.33	0
<i>Candida deformans</i>	100	100	0	0	0
<i>Candida pseudoglaebosa</i>	100	100	0	0	0
<i>Candida saitoana</i>	100	100	0	0	0
<i>Candida variabilis</i>	100	93.75	6.25	0	0
<i>Candida versatilis</i>	100	100	0	0	0
<i>Chrysonilia sitophila</i>	100	100	0	0	0

Table 3. Cont.

Species	Overall correct (%) ⁽¹⁾	Single choice (%)	Low discrimination (%)	No identification (%)	Discordant (%)
<i>Chrysosporium keratinophilum</i>	100	100	0	0	0
<i>Cladosporium allicinum</i>	100	100	0	0	0
<i>Cladosporium bruhnei</i>	100	100	0	0	0
<i>Cladosporium macrocarpum</i>	85.71	42.86	42.86	7.14	7.14
<i>Colletotrichum karsti</i>	90	90	0	10	0
<i>Cunninghamella elegans</i> *	100	100	0	0	0
<i>Cystobasidium minutum</i> *	97.44	87.18	10.26	2.56	0
<i>Engyodontium album</i>	94.12	94.12	0	5.88	0
<i>Eupenicillium lapidosum</i>	100	100	0	0	0
<i>Exophiala bergeri</i>	96.55	96.55	0	1.72	1.72
<i>Exophiala lecanii-corni</i>	100	100	0	0	0
<i>Exophiala oligosperma</i>	100	100	0	0	0
<i>Exophiala pisciphila</i>	100	100	0	0	0
<i>Exophiala psychrophila</i>	100	100	0	0	0
<i>Filobasidium magnum</i>	100	100	0	0	0
<i>Fusarium verticillioides</i> *	73.53	73.53	0	18.18	0
<i>Geosmithia swiftii</i> *	100	100	0	0	0
<i>Hannaella luteola</i>	81.82	81.82	0	18.18	0
<i>Helicostylum elegans</i>	100	100	0	0	0
<i>Hortaea werneckii</i>	100	100	0	0	0
<i>Hyphopichia pseudoburtonii</i>	100	100	0	0	0
<i>Isomucor fuscus</i>	100	100	0	0	0
<i>Kazachstania exigua</i>	100	100	0	0	0
<i>Kazachstania unispora</i>	100	100	0	0	0
<i>Lachancea kluyveri</i>	100	100	0	0	0
<i>Linnemannia hyalina</i>	100	100	0	0	0
<i>Linnemannia zychae</i>	100	100	0	0	0
<i>Microdochium nivale</i> *	100	100	0	0	0
<i>Mucor mucedo</i>	100	100	0	0	0
<i>Mucor piriformis</i>	95	95	0	5	0
<i>Nigrospora oryzae</i>	100	100	0	0	0
<i>Nigrospora sphaerica</i>	100	100	0	0	0
<i>Paraphyton cookei</i>	100	100	0	0	0
<i>Penicillium bifforme</i>	100	87.72	12.28	0	0
<i>Penicillium charlesii</i>	90	80	10	10	0
<i>Penicillium corylophilum</i> *	100	100	0	0	0
<i>Penicillium fellutanum</i>	100	100	0	0	0
<i>Penicillium funiculosum</i>	100	93.75	6.25	0	0
<i>Penicillium glandicola</i>	100	100	0	0	0
<i>Penicillium griseofulvum</i>	100	100	0	0	0
<i>Penicillium islandicum</i>	100	100	0	0	0
<i>Penicillium janczewskii</i>	100	100	0	0	0
<i>Penicillium janthinellum</i>	100	100	0	0	0
<i>Penicillium macrosporum</i> *	98.57	98.57	0	1.43	0
<i>Penicillium olsonii</i>	100	100	0	0	0
<i>Penicillium purpurogenum</i>	93.33	93.33	0	3.33	3.33
<i>Penicillium rubens</i>	100	96.43	3.57	0	0
<i>Penicillium rugulosum</i>	100	100	0	0	0
<i>Penicillium ubiquestum</i>	95.65	95.65	0	4.35	0
<i>Phoma pinodella</i>	100	100	0	0	0
<i>Pichia occidentalis</i>	100	100	0	0	0
<i>Rhinocladiella similis</i>	92.86	92.86	0	7.14	0
<i>Rhizomucor pusillus</i>	100	100	0	0	0
<i>Saccharomyces cariocanus</i>	100	100	0	0	0
<i>Saccharomyces uvarum</i>	100	100	0	0	0
<i>Saccharomycopsis fibuligera</i>	100	100	0	0	0
<i>Schizophyllum commune</i>	100	100	0	0	0
<i>Scopulariopsis asperula</i> *	100	100	0	0	0
<i>Scopulariopsis candida</i>	100	100	0	0	0
<i>Scopulariopsis flava</i>	100	100	0	0	0
<i>Sporobolomyces roseus</i>	100	100	0	0	0

Table 3. Cont.

<i>Stachybotrys chartarum</i>	100	100	0	0	0
<i>Starmerella etchellsii</i>	100	100	0	0	0
<i>Syncephalastrum racemosum</i>	100	100	0	0	0
<i>Thamnidium elegans</i>	100	100	0	0	0
<i>Trichoderma atroviride</i>	100	0	100	0	0
<i>Trichoderma harzianum</i> *	100	100	0	0	0
<i>Trichoderma viride</i> *	100	0	100	0	0
<i>Trigonopsis californica</i>	100	100	0	0	0
<i>Verticillium albo-atrum</i>	100	0	100	0	0
<i>Verticillium dahliae</i>	97.37	97.37	0	2.63	0
<i>Verticillium nonalfalfae</i>	100	0	100	0	0
<i>Wallemia muriae</i>	100	100	0	0	0
<i>Xeromyces bisporus</i>	96.67	96.67	0	3.33	0
<i>Zygorulasporea florentina</i>	100	100	0	0	0
<i>Zygorulasporea mrakii</i>	100	100	0	0	0
<i>Aspergillus brasiliensis</i> *	100	100	0	0	0
<i>Aspergillus luchuensis</i> (ex <i>Aspergillus coreanus</i>)	96.97	96.97	0	3.03	0
<i>Aspergillus eucalypticola</i>	100	0	100	0	0
<i>Aspergillus niger</i> (ex <i>Aspergillus</i> <i>foetidus</i>) **	100	100	0	0	0
<i>Aspergillus niger</i> (ex <i>Aspergillus</i> <i>lacticoffeatus</i>) **	100	100	0	0	0
<i>Aspergillus luchuensis</i> (ex <i>Aspergillus piperis</i>)	77.27	0	77.27	4.55	18.18
<i>Aspergillus niger</i> **	100	100	0	0	0
<i>Aspergillus tubingensis</i> *	91.15	91.15	0	4.42	4.42
<i>Aspergillus vadensis</i>	100	100	0	0	0
<i>Bisifusarium allantoides</i>	100	100	0	0	0
<i>Bisifusarium bisepatum</i>	100	100	0	0	0
<i>Bisifusarium delphinoides</i>	100	0	100	0	0
<i>Bisifusarium dimerum</i> **	100	100	0	0	0
<i>Bisifusarium domesticum</i> **	100	100	0	0	0
<i>Bisifusarium lunatum</i>	100	100	0	0	0
<i>Bisifusarium nectrioides</i>	100	0	100	0	0
<i>Bisifusarium penicillioides</i>	100	100	0	0	0
<i>Bisifusarium penzigii</i>	100	100	0	0	0
<i>Colletotrichum acutatum</i>	100	100	0	0	0
<i>Colletotrichum fioriniae</i>	100	100	0	0	0
<i>Colletotrichum godetiae</i>	90.48	90.48	0	9.52	0
<i>Colletotrichum lupini</i>	100	100	0	0	0
<i>Colletotrichum nymphaeae</i>	100	100	0	0	0
<i>Colletotrichum fructicola</i>	100	100	0	0	0
<i>Colletotrichum gloeosporioides</i>	100	100	0	0	0
<i>Colletotrichum musae</i>	100	100	0	0	0
<i>Colletotrichum siamense</i>	82.14	0	82.14	17.86	0
<i>Colletotrichum tropicale</i>	73.68	0	73.68	26.32	0
<i>Mucor bainieri</i>	100	0	100	0	0
<i>Mucor circinelloides</i> **	100	100	0	0	0
<i>Mucor ctenidius</i>	100	100	0	0	0
<i>Mucor griseocyanus</i>	100	100	0	0	0
<i>Mucor janssenii</i>	100	100	0	0	0
<i>Mucor lusitanicus</i>	100	0	100	0	0
<i>Mucor ramosissimus</i>	100	0	100	0	0
<i>Mucor variicolumellatus</i>	100	0	100	0	0
<i>Mucor velutinosus</i> *	95	95	0	5	0
<i>Thamnidium anomalum</i>	100	100	0	0	0

* Species already present in the database for which additional strains were integrated. ** Species from species complexes already present in the database. ⁽¹⁾ Single choice stands for spectra identified to the correct species, low discrimination corresponds to spectra which matched with different species including the correct one and the overall correct percentage results of the addition of single choice and low discrimination percentages.

The cross-validation approach is the first method to evaluate performance and highlight possible cross-identifications. To go further in the evaluation of identification performance, an external validation was conducted with strains not included in the database.

3.1.2. Database Validation

The database was challenged using an external dataset. Overall, the external validation performances were the following for the species present in the database: 89.42% spectra were correctly identified, 8.65% were not identified and 1.92% were misidentified. For 62 out of 75 strains for which species were represented in the database, all acquired spectra showed expected results, i.e., a correct identification (Table 4). Species which did not yield satisfactory results were *Arthrographis kalrae*, *A. creber*, *A. jensenii*, *Chrysosporium keratinophilum*, *Engyodontium album*, the *Fusarium solani* complex, *Hortaea werneckii*, *Mucor plumbeus*, *Mucor piriformis*, *Penicillium aurantiogriseum*, *P. bifforme* and *Zygorhynchus mrakii*. Spectra from these species were either unidentified or inappropriately identified. Among those, only six species, i.e., *A. kalrae*, *A. creber*, *C. keratinophilum*, *C. gloeosporioides*, the *F. solani* complex, *M. piriformis*, *P. aurantiogriseum*, had less than 60% correctly identified spectra. Noteworthy, erroneously identified spectra were assigned to the correct genus. Indeed, spectra from *A. creber* were misidentified as *Aspergillus versicolor*.

Table 4. Performance evaluation of the database by external validation.

	Species (number of strains)	Number of spectra	Number of correct identification	Number of no identification	Number of misidentification
Species present in the database					
	<i>Alternaria brassicicola</i>	1	2		
	<i>Arthrographis kalrae</i>	1	4	2	
	<i>Aspergillus amoenus</i>	1	4		
	<i>Aspergillus cibarius</i>	1	4		
	<i>Aspergillus creber</i>	2	12	6	4 (<i>Aspergillus versicolor</i>)
	<i>Aspergillus hiratsukae</i>	2	6	6	
	<i>Aspergillus jensenii</i>	1	3	2	1
	<i>Aspergillus tubingensis</i>	1	2	2	
	<i>Aureobasidium pullulans</i>	1	2	2	
	<i>Bisifusarium bisseptatum / penzigii</i>	1	4	4	
	<i>Botrytis cinerea</i>	2	4	4	
	<i>Candida famata</i>	1	2	2	
	<i>Candida guilliermondii</i>	1	2	2	
	<i>Candida hellenica</i>	1	2	2	
	<i>Candida pulcherrima</i>	1	2	2	
	<i>Chrysonilia sitophila</i>	2	10	10	
	<i>Chrysosporium keratinophilum</i>	1	4	2	2
	<i>Cladosporium cladosporioides</i>	3	6	6	
	<i>Cladosporium oxysporum</i>	1	2	2	
	<i>Cladosporium ramotenellum</i>	1	2	2	
	<i>Colletotrichum lupini</i>	2	8	8	
	<i>Engyodontium album</i>	1	6	5	1
	<i>Eupenicillium lapidosum</i>	1	4	4	
	<i>Exophiala dermatitidis</i>	1	2	2	
	<i>Fusarium proliferatum</i>	2	4	4	
	<i>Fusarium sambucinum</i>	1	2	2	
	<i>Fusarium solani</i> complex	1	2	1	1
	<i>Geotrichum candidum</i>	1	2	2	
	<i>Hortaea werneckii</i>	3	6	5	1
	<i>Kloeckera apiculata</i>	1	2	2	
	<i>Microdochium nivale</i>	1	5	5	

Table 4. Cont.

	Species (number of strains)	Number of spectra	Number of correct identification	Number of no identification	Number of misidentification
	<i>Mucor circinelloides</i>	1	2		
	<i>Mucor plumbeus</i>	2	4	3	1
	<i>Mucor piriformis</i>	1	2	2	
	<i>Papiliotrema laurentii</i>	1	2	1	1
	<i>Penicillium adametzioides</i>	1	2	2	
	<i>Penicillium aurantiogriseum</i>	1	2	2	2
	<i>Penicillium aurantiogriseum var. polonicum</i>	1	2	2	
	<i>Penicillium biforme</i>	3	12	10	2
	<i>Penicillium brevicompactum</i>	1	2	2	
	<i>Penicillium citrinum</i>	1	2	2	
	<i>Penicillium crustosum</i>	2	4	4	
	<i>Penicillium digitatum</i>	1	2	2	
	<i>Penicillium expansum</i>	1	2	2	
	<i>Penicillium glabrum</i>	1	2	2	
	<i>Penicillium italicum</i>	1	2	2	
	<i>Penicillium olsonii</i>	2	8	8	
	<i>Penicillium paneum</i>	1	2	2	
	<i>Penicillium rubens</i>	2	8	8	
	<i>Penicillium solitum</i>	1	2	2	
	<i>Phoma glomerata</i>	2	6	6	
	<i>Rhizopus stolonifer</i>	1	2	2	
	<i>Rhodotorula mucilaginosa</i>	1	2	2	
	<i>Sporobolomyces roseus</i>	1	2	2	
	<i>Trichoderma harzianum</i>	1	2	2	
	<i>Trichoderma viride/ghanense</i>	1	2	2	
	<i>Verticillium nonalfalfae</i>	1	2	2	
	<i>Zygotrypastrum mrakii</i>	1	4	2	2
Species absent in the database					
	<i>Cladosporium snafimbriatum</i>	1	2		2 (<i>Cladosporium allicinum</i> and <i>Cladosporium macrocarpum</i>)
	<i>Mucor brunneogriseus</i>	1	2	2	
	<i>Rhodotorula babjevae</i>	1	2	2	

Concerning the three strains belonging to species that were not part of the database, all acquired spectra for two of them, i.e., *Mucor brunneogriseus* and *Rhodotorula babjevae*, yielded a “no identification” result, while spectra from *Cladosporium snafimbriatum* were identified as *C. allicinum*/*C. macrocarpum* (low discrimination). It is worth mentioning that *C. snafimbriatum*, a newly described species, is a member of the *Cladosporium herbarum* complex and is also closely related to *C. allicinum* and *C. macrocarpum* [41].

3.2. Performance Evaluation of MALDI-TOF MS for Species Complex Differentiation

The ability of MALDI-TOF MS for discriminating species within five species complexes, i.e., *Aspergillus* series *Nigri*, *Colletotrichum acutatum* complex, *Mucor circinelloides* complex, *Colletotrichum gloeosporioides* complex and *Bisifusarium dimerum* complex, was evaluated using non-supervised (t-SNE) and supervised approaches (cross-validation). All recognized species within these species complexes were analyzed using MALDI-TOF MS with the exception of *Colletotrichum asianum* and *Bisifusarium tonghuanum* that could not be obtained from international culture collections. The spectra from the five species complexes are displayed on t-SNE maps (Figure 1). As shown in Figure 1A and Supplementary Figure S1, some species from the *Aspergillus* series *nigri*, such as *A. brasiliensis*, *A. tubingensis*, *A. luchuensis* (ex ‘*Aspergillus coreanus*’) and *A. vadensis*, were distinguishable

with well-grouped spectra according to their respective species. Spectra from *A. niger* and those from '*Aspergillus lacticoffeatus*' and '*Aspergillus foetidus*' which are now considered as synonyms of *A. niger* were grouped together which is consistent with Bian et al. [33]. Noteworthy, the effect of cultivation time was visible for two species, i.e., '*Aspergillus piperis*' (synonym of *A. luchuensis*) and *A. eucalypticola*. For '*Aspergillus piperis*' (synonym of *A. luchuensis*), spectra obtained after 8 d were grouped on the upper quadrant, one group on the left and one on the right, while the 2-day spectra were on the lower quadrant. The same results were also observed for *A. eucalypticola* for which 2-day spectra were at the bottom of the lower quadrant, whereas 8-day spectra were at the top of the upper quadrant.

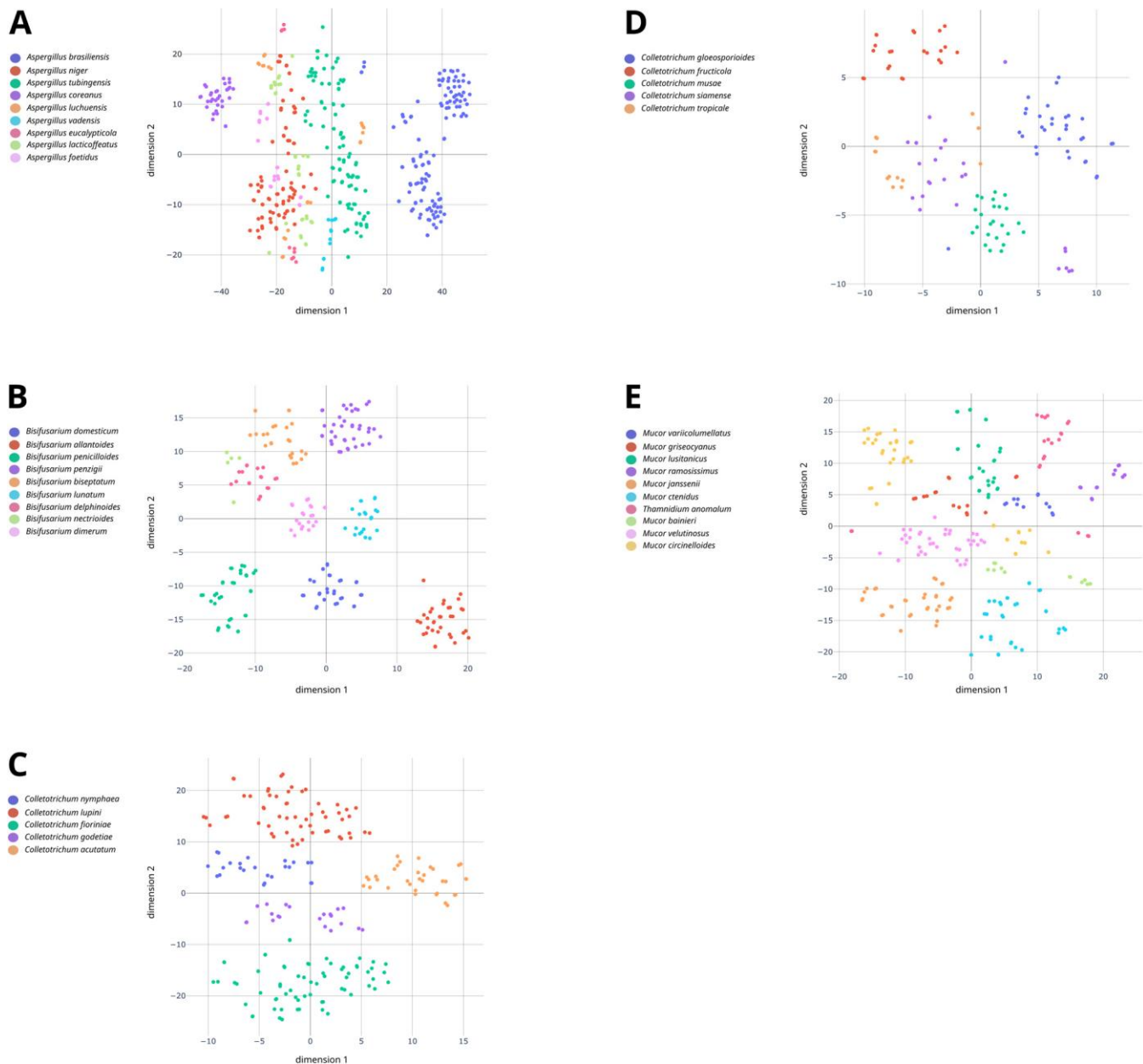


Figure 1. A two-dimensional t-SNE map displaying the spectra from the *Aspergillus* series *nigri* (A), the *Bisufusarium dimerum* complex (B), the *Colletotrichum acutatum* complex (C), the *Colletotrichum gloeosporioides* complex (D) and the *Mucor circinelloides* complex (E) obtained through MALDI-TOF MS. Spectra are colored according to the respective species to which they belong.

As shown in Figure 1B and Supplementary Figure S2, spectra from the different species of the *Bisufusarium dimerum* complex were also quite well separated. *B. allantoides*,

B. domesticum and *B. penicillioides* spectra were grouped on the lower quadrant of the t-SNE map, whereas the remaining species were grouped on the upper quadrant (Figure 1B). Spectra from *B. nectrioides* and *B. delphinooides* appeared to be more closely related on the t-SNE map (Figure 1B) which was also confirmed on the spectral similarity dendrogram (Supplementary Figure S3, similarity = 65%).

As shown in Figure 1C,D, species within each of the *C. acutatum* and *C. gloeosporioides* complexes demonstrated clear intra-complex separations even though they shared a relatively high-level similarity of over 60% in both cases (Supplementary Figures S3 and S4). Concerning the *C. acutatum* complex, spectra for all of the five species analyzed, i.e., *Colletotrichum nymphaeae*, *Colletotrichum lupini*, *Colletotrichum fioriniae*, *Colletotrichum godetiae* and *C. acutatum*, were well clustered and separated for each species (Figure 1C). As for the *C. gloeosporioides* complex, two species could be easily distinguished on the t-SNE map, i.e., *Colletotrichum fructicola* and *C. gloeosporioides* (Figure 1D). Their scatterplots were distant from each other and from all the other scatterplots. The remaining species, namely *Colletotrichum musae*, *C. siamense* and *C. tropicale*, were mostly grouped on the lower left quadrant. The *C. musae* spectra were well clustered, while the *C. siamense* and *C. tropicale* spectra were interspersed. The spectra for *C. siamense* were mostly present between the two clusters of *C. tropicale* spectra.

As shown in Figure 1E and Supplementary Figure S5, species from the *M. circinelloides* complex were also well separated, namely *Mucor variicolumellatus*, *Mucor lusitanicus*, *Mucor ramosissimus*, *Mucor janssenii*, *Mucor ctenidius*, *Mucor velutinosus* and *Mucor griseocyanus*. Two spectra from the latter species were separated from the others. They were both obtained after cultivation for 2 d on MEA (Oxoid), so it was assumed that it was linked to this specific condition. The impact of incubation time was also noticeable for *Mucor bainieri*, *M. circinelloides* and *Thamnidium anomalum*. For instance, *M. bainieri* spectra at 2 d post incubation were on the left of the right quadrant, while the spectra at 8 d post incubation were on the right of the left quadrant. The same results, but to a much lower extent, were also observed for *M. circinelloides* and *T. anomalum*. Indeed, the spectra of each species were grouped together, but part of the spectra obtained after a 2-day incubation were typically separated from those obtained after an 8-day incubation.

Spectra from the different species of the tested species complex were integrated into the bioMérieux spectral database, and identification performances were assessed by cross-validation (Table 3). The *Aspergillus* series Nigri, comprising currently six species, yielded levels of correct identification ranging from 77.27% to 100% with an overall correct identification of 100% for four species, i.e., *A. brasiliensis*, *A. eucahypticola*, *A. niger* including isolates of '*A. foetidus*' and '*A. lacticoffeatus*' and *A. vadensis*. For the *B. dimerum* complex, which includes nine species, a performance of 100% correct identification was reached. Concerning the *C. acutatum* complex, correct identification rates ranged from 90.48% to 100% where four out of five species were found to yield 100% correct identification. Good performances were also achieved for the *C. gloeosporioides* complex with correct identification levels ranging from 73.68% to 100% and spectra from three out of five species yielding 100% correct identification. Finally, for the *M. circinelloides* complex, correct spectra identification ranged from 95% to 100%, and nine of the ten species had a 100% correct identification level.

4. Discussion

4.1. Performance Estimation by Cross-Validation and Database Validation

In a previous study, Quéro et al. [42] complemented the VITEK[®] MS database using 136 species encountered in the food and feed industry demonstrating the importance of an updated database for fungal identification. In the present study, the VITEK[®] MS Knowledge Base was further reinforced with 119 new selected species and 20 species already present in the database for which improvements were made. The overall cross-validation performance was 96.55% with 3.1% unidentified and 0.35% misidentified.

Overall, 97.12% of the species examined under this study (Table 3) had a correct identification rate ranging from 80% to 100%. Additionally, 109 out of 139 species yielded an overall correct identification rate of 100%, accounting for 78.42% of the species examined. However, the correct identification rates of four species fell below 80%, i.e., *A. fischeri* (76.92%), *A. luchuensis* (77.27%), *C. tropicale* (73.68%) and *F. verticillioides* (73.53%). This lower identification performance could be linked to cross-identifications between closely related species in the database. For instance, *A. luchuensis* had a level of discordant spectra of 18.18%, which were identified as the “Niger complex” rather than at the species level. *Aspergillus luchuensis* is indeed one of the species of the Niger clade. Species within this clade share a high similarity with one another, and it is difficult to distinguish them despite the use of multigenic DNA barcoding [33,35]. For *F. verticillioides*, we have no clear explanation for this result that was also previously observed by Quero et al. [18]. The fact that 25% of spectra yielded no identification during cross-validation may be caused by the high genetic diversity of *F. verticillioides* at the intra-species level and/or the existence of yet-to-be-identified cryptic species [43]. Therefore, the enrichment of the database with spectra of a larger diversity of strains at the population level could improve identification performance for this species.

The cross-validation results provided an estimation of the database performance. An external validation using strains not included in the database was necessary to assess the identification performance. Considering the results for all tested strains, it is promising that 90.48% spectra were correctly assigned as expected. The misidentified spectra were ascribed to either the closely related species from the same genus or from the same species complex or both. For instance, spectra from *C. snafimbriatum* were identified as *C. allicinum* and *C. macrocarpum*, two closely related species within the same species complex [41]. To address this, the database could be expanded in a future version to encompass more species from the *C. herbarum* complex, including *C. snafimbriatum*. Noteworthy, the external and cross-validation results were consistent. *A. creber*, for example, had an overall identification performance of 89.19% during cross-validation with 8.11% spectra showing low discrimination with *Aspergillus versicolor*. The same result was also found during external validation. This issue could be addressed by adding more strains from the species of the *Aspergillus* series *versicolores* to optimize the identification accuracy of the spectral database. Noteworthy, a simplified classification of the series *versicolores* with a lower number of cryptic species was recently proposed by Sklenář et al. [44], leading to the definition of only four species instead of seventeen. As mentioned by Sklenář et al. [44], the use of this classification for spectral database construction may also improve identification accuracy for this series.

4.2. Performance Evaluation of MALDI-TOF MS for Species Complex Differentiation

In total, five species complexes were studied using non-supervised (t-SNE) and supervised approaches. The t-SNE method was used as an unsupervised technique to study closely related species and to assess the discriminatory ability of MALDI-TOF MS. As previously seen, this projection enabled us to differentiate species within the same complex despite a high spectral similarity which can be a struggle using only dendrograms. In fact, this non-linear projection technique enables the visualization of high-dimensional data in a lower-dimensional map and discerns specificity that were not perceptible in other arrangements [37,38].

Despite being phylogenetically and genetically close, four out of the six species of the *Aspergillus* series *Nigri* had an overall correct identification of 100% in cross-validation, and two species were above 90%, i.e., *A. luchuensis* (ex ‘*A. coreanus*’) (96.67%) and *A. tubingensis* (91.15%). *A. luchuensis* (ex ‘*Aspergillus piperis*’) was the only one of the sections with a correct identification level under 80%. In Bian et al. [33], the species-level identification of *Aspergillus* section *Nigri* is considered problematic, if not impossible, even using techniques such as DNA sequencing or MALDI-TOF MS. Yet, following the redefined *Aspergillus* series *Nigri* proposed by Bian et al. [33], a cross-validation performance above

90% was obtained for eight out of the nine species. This demonstrates an improvement in intra-specific differentiation within this section using MALDI-TOF MS, which could address the current difficulty of identifying these hazardous mycotoxin producers.

Secondly, *Colletotrichum* complexes, causative agents of anthracnosis, are responsible for food waste, resulting in an important economic impact. They mainly encompass phytopathogenic species that affect a wide variety of hosts causing considerable crop losses. For instance, two prominent complexes, *C. acutatum* and *C. gloeosporioides*, are responsible for fruit crop infections worldwide, leading to massive plant necrosis [45,46]. Because of their similar characteristics, they could be difficult to differentiate. In the present study, MALDI-TOF MS proved to be a good alternative to molecular techniques to discriminate these species within complexes. The cross-validation results for the *C. acutatum* complex showed 90.48% to 100% of spectra being correctly identified with four out of five species having 100% correct identification. The *C. gloeosporioides* species performance levels varied from 73.68% to 100% with three of them achieving 100% correct identification. This identification performance is promising and could significantly enhance disease management strategies and future management outlook [45,46]. The t-SNE method also showed separated scatterplots for each *C. acutatum* complex species. One strain of *C. godetiae* appeared close to the *C. fiorinae* scatterplots which confirmed the results obtained by cross-validation. However, the phylogenetic data did not show a taxonomic misassignment for this distant strain. The apparent vicinity of *C. acutatum*, *C. nymphaeae* and *C. lupini* on one part and *C. fiorinae* and *C. godetiae* on the other part is also consistent with the scientific literature [47].

Thirdly, the *B. dimerum* and *M. circinelloides* complexes were those with the best identification performances, i.e., 100% for all species and 100% correct identification level for every species except *M. velutinosus* (95%), respectively. Interestingly, these complexes are relevant to differentiate for different reasons. Indeed, the *B. dimerum* complex, which belongs to the *Nectriaceae* family, comprises either plant pathogens, species responsible for opportunistic infections and food spoilage but also a species (*B. domesticum*) voluntarily used by cheesemakers to prevent cheese organoleptic defects (stickiness defect) [48–51]. Noteworthy, the *B. dimerum* complex which reached 100% correct identification to the species level in cross-validation shows clear clusters in two-dimensional projection and a two-group organization. Those results are in agreement with the phylogenetic analysis conducted by Savary et al. [50]. The result of the present study also confirmed the proximity of *B. nectrioides* and *B. delphinoides*.

The *M. circinelloides* species complex includes saprophytic species responsible for food spoilage that are also known as opportunistic pathogens responsible for mucormycosis in immunocompromised patients [52,53]. Different studies have already demonstrated clear differences in virulence [54] and antifungal susceptibilities [55] among species (or forms formerly) within the complex. Given these concerning issues and the identification performance achieved in this study, MALDI-TOF MS can be a powerful asset for discriminating these species that may have varying ecologies and virulence levels. Among this complex, some species were rather well separated on the t-SNE map, whereas some species were not, and it seems to be linked to growth media and incubation time. Nevertheless, it did not impact identification performances during cross-validation. Similar results have been reported by Quérou et al. [18] for other species, i.e., *A. flavus*, *Aureobasidium pullulans* and *P. expansum*. These peculiar cases are important to keep in the VITEK[®] MS database because it adds spectral diversity and thus allows us to build a robust database.

5. Conclusions

In the present study, the existing VITEK MS database was extended with food-relevant fungal species as well as species belonging to species complexes. It appeared that MALDI-TOF MS was a powerful tool to accurately identify these fungal species as well as to discriminate species within species complexes. These results emphasize the importance of continuously enhancing the database by incorporating relevant species and species complexes and taking into account the continuous evolution and progression of fungal taxonomy.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/jof10070456/s1>, Figure S1: A dendrogram based on spectral similarity between the different strains of *Aspergillus* series *nigri* included in the VITEK MS[®] Knowledge Base. Figure S2: A dendrogram based on spectral similarity between the different strains of *Bisifusarium dimerum* complex included in the VITEK MS[®] Knowledge Base. Figure S3: A dendrogram based on spectral similarity between the different strains of the *Colletotrichum acutatum* complex included in the VITEK MS[®] Knowledge Base. Figure S4: A dendrogram based on spectral similarity between the different strains of the *Colletotrichum gloeosporioides* complex included in the VITEK MS[®] Knowledge Base. Figure S5: A dendrogram based on spectral similarity between the different strains of the *Mucor circinelloides* complex included in the VITEK MS[®] Knowledge Base.

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Conflicts of Interest: N.R., V.G., V.M., S.A., V.C., M.D.A., G.P. and R.B. are employees of bioMérieux, a company developing and selling in vitro diagnostic assays including the VITEK MS used in this study. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

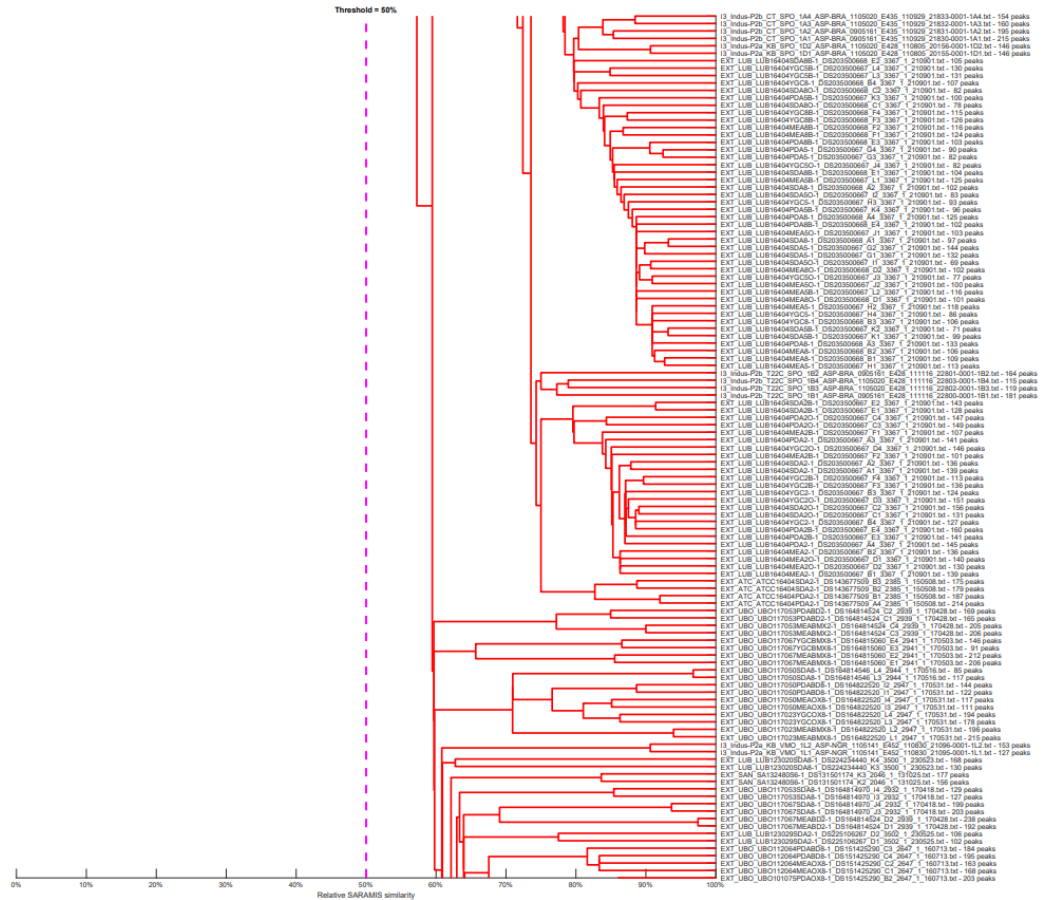
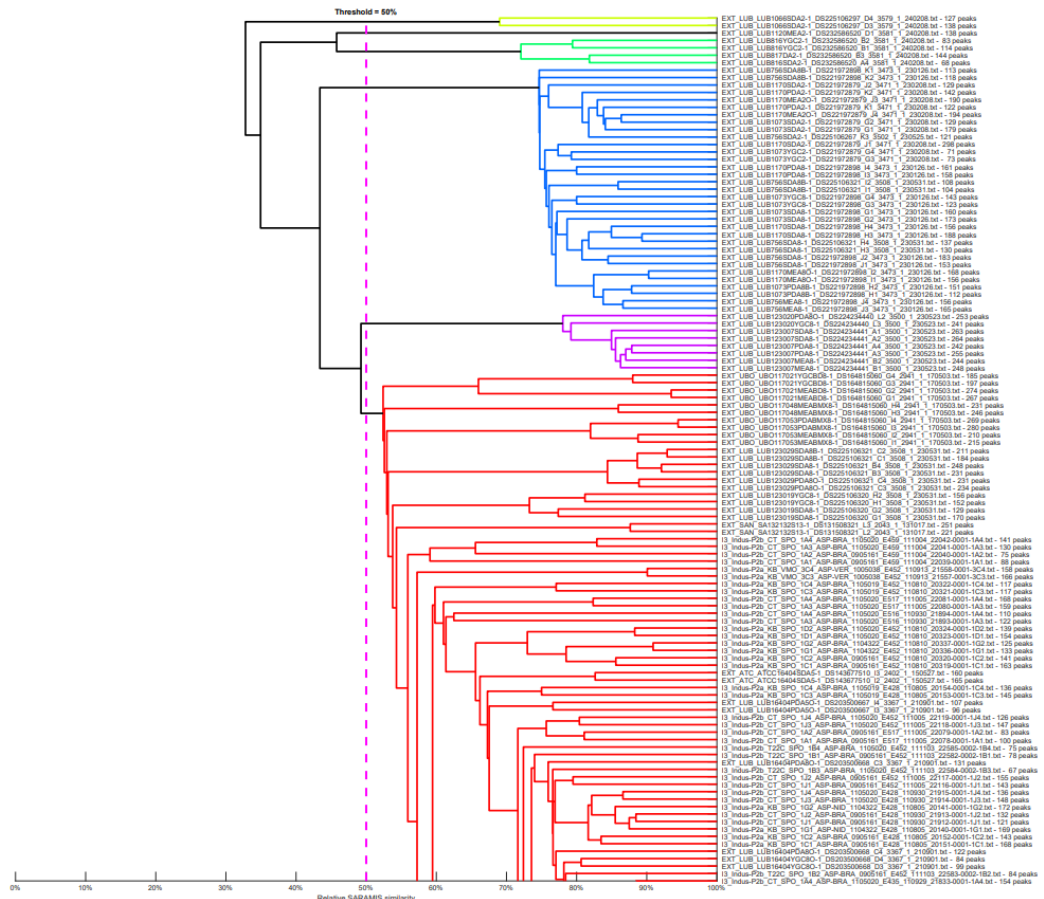
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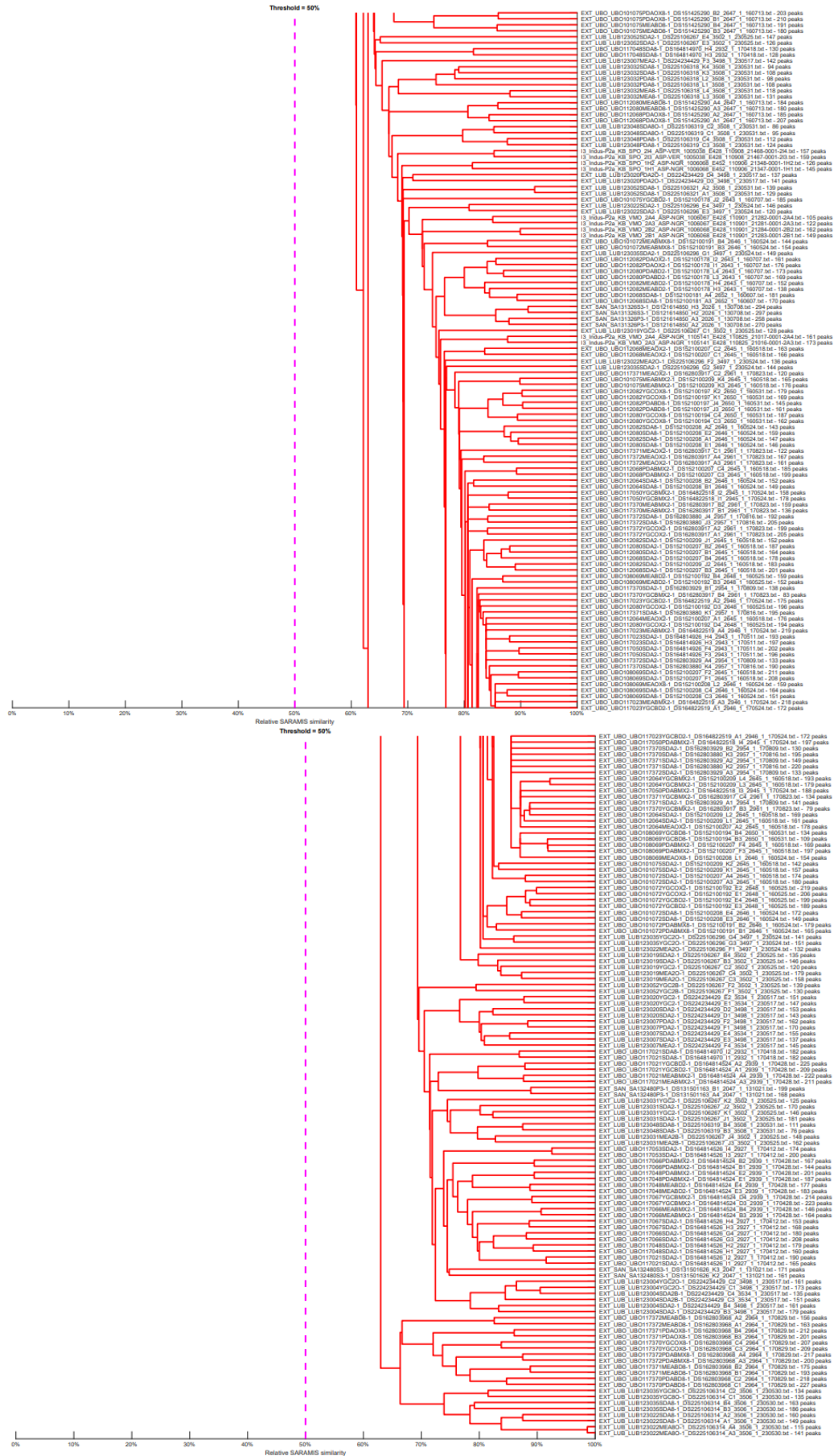


Figure S1: Dendrogram based on spectral similarity between the different strains of *Aspergillus* series *nigri* included in the VITEK MS® Knowledge Base

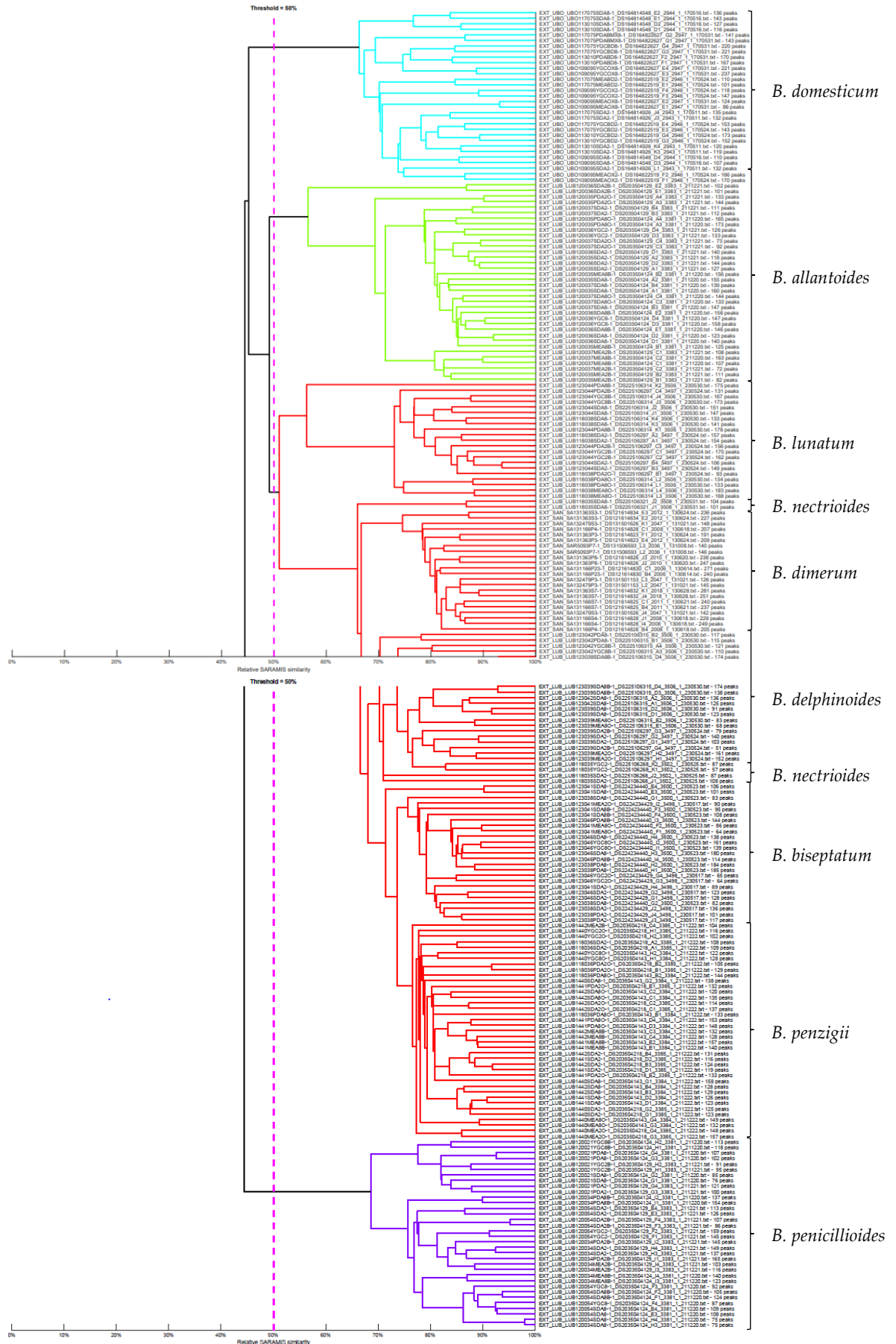


Figure S2: Dendrogram based on spectral similarity between the different strains of *Bisfusarium dimerum* complex included in the VITEK MS® Knowledge Base

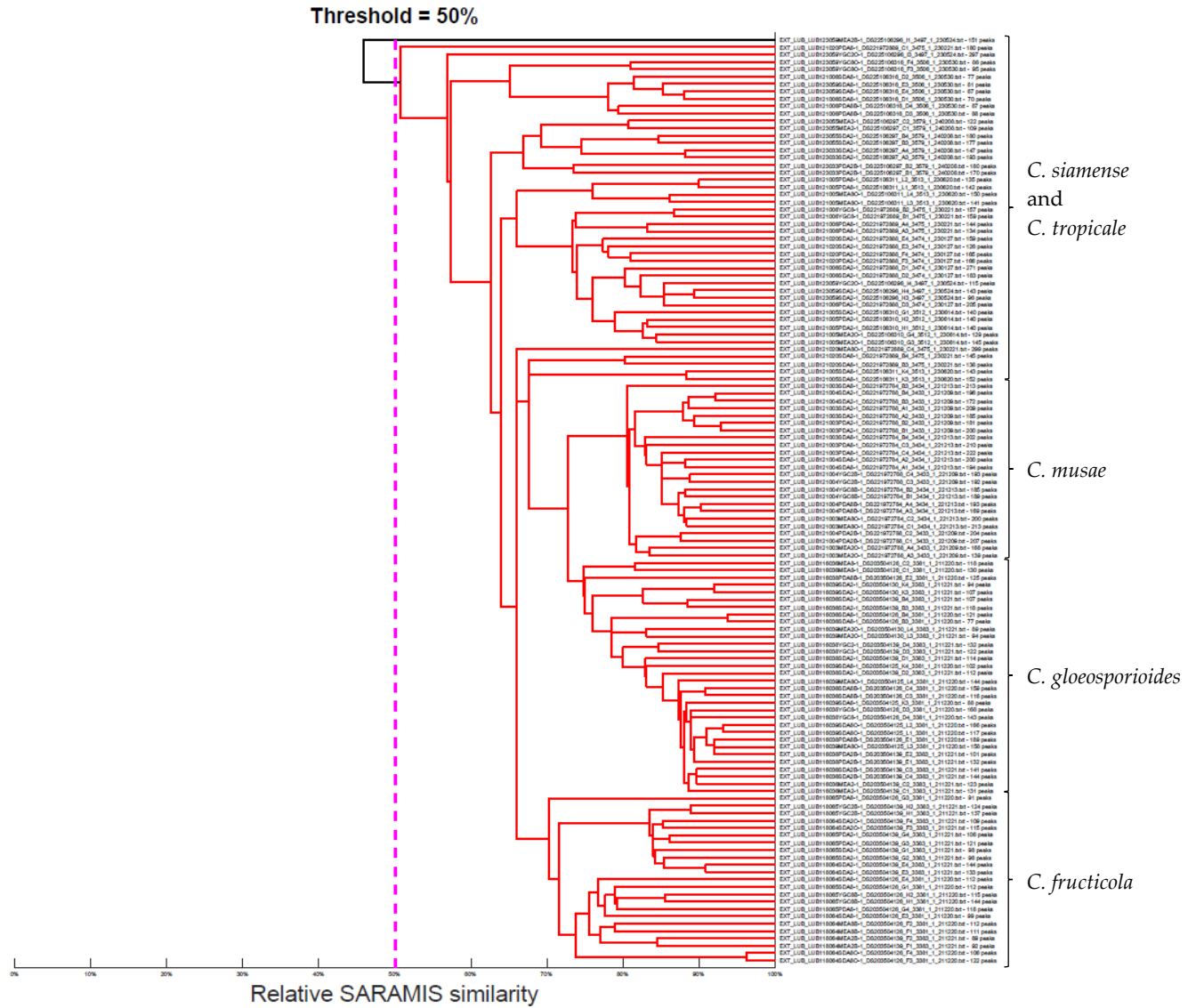


Figure S4: Dendrogram based on spectral similarity between the different strains of *Colletotrichum gloeosporioides* complex included in the VITEK MS® Knowledge Base

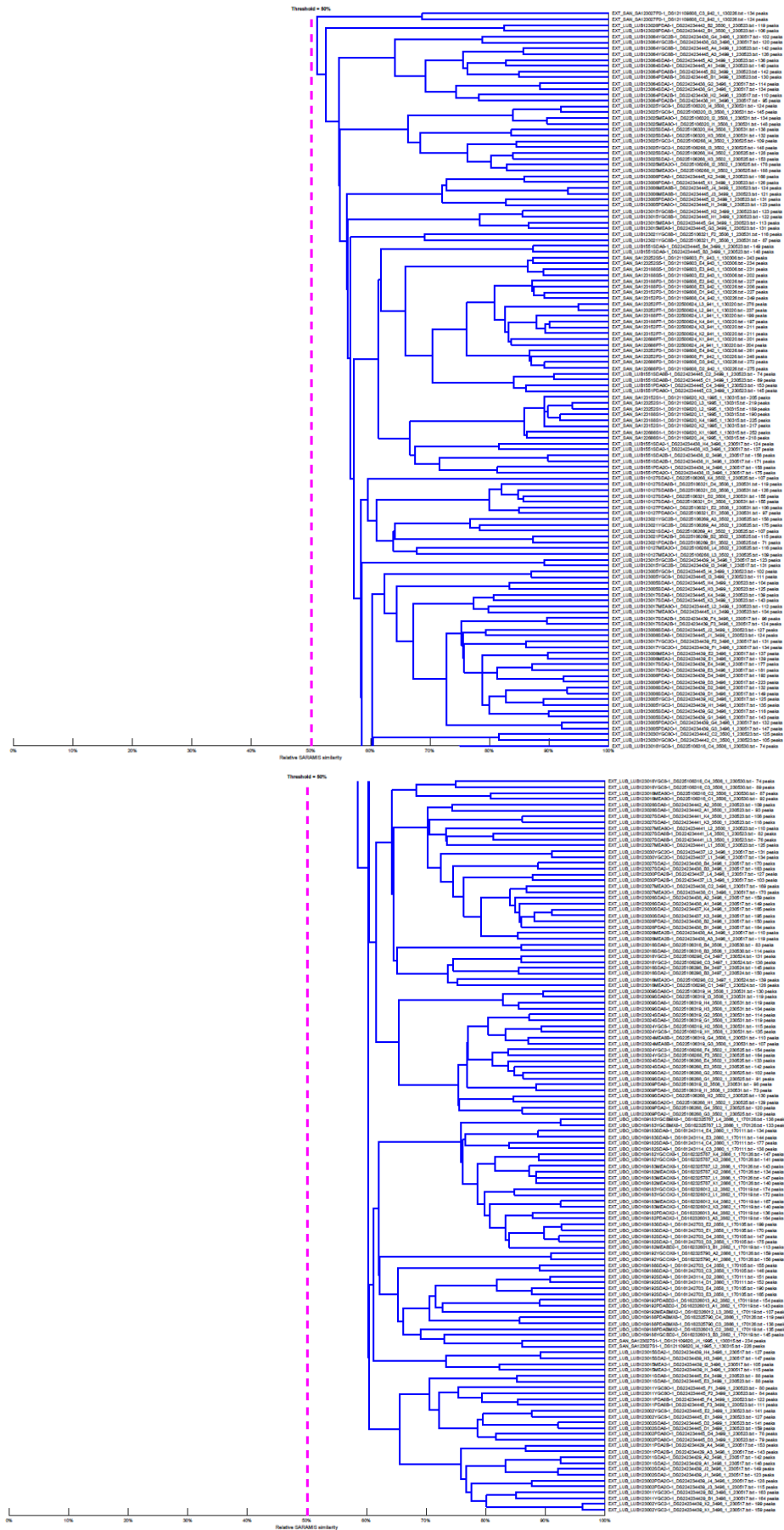


Figure S5: Dendrogram based on spectral similarity between the different strains of *Mucor circinelloides* complex included in the VITEK MS® Knowledge Base

III. Résultats supplémentaires

1. Essais de compatibilité de milieux pour la construction de la base de données levures VITEK® MS KB 3.4

En complément des travaux présentés dans cette étude, des essais préliminaires ont été menés afin de déterminer la compatibilité de milieux de culture couramment utilisés en industrie pour l'analyse de levures d'intérêt agro-alimentaire par spectrométrie de masse et le développement de la base de données. En effet, ce point n'avait pas été abordé lors du précédent projet qui s'était concentré sur les champignons filamenteux.

A cet effet, cinq à six souches appartenant à quatre espèces levuriennes fréquemment retrouvées en industrie ont été sélectionnées (i.e. *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii* et *Candida intermedia*), soit 21 souches au total. Ces souches ont été cultivées sur milieu SDA, considéré comme milieu de référence, ainsi que sur six milieux couramment utilisés en industrie dont nous souhaitons estimer la compatibilité pour l'analyse par spectrométrie de masse MALDI-TOF. Les géloses considérées sont, d'une part, les géloses PDA, YGC et MEA (bioMérieux, Marcy l'Etoile, France), toutes utilisées pour la culture, la recherche et le dénombrement des levures et des moisissures dans les matrices alimentaires, et d'autre part, les milieux, Oxytétracycline Glucose Agar (OGA) recommandé par la norme Agence Française de Normalisation (AFNOR) pour le dénombrement des levures et des moisissures dans les produits laitiers et plus généralement dans les matrices alimentaires, Dichloran Rose Bengale Chloramphénicol (DRBC ; Oxoid – Thermo Fischer Scientific, Dardilly, France) utilisé dans l'industrie pour le dénombrement des levures et moisissures viables dans les produits destinés à l'alimentation humaine et animale et Dichloran Glycérol (DG18 ; Oxoid – Thermo Fischer Scientific, Dardilly, France) utilisé dans l'industrie pour le dénombrement des levures et des moisissures xérophiles.

Les souches sélectionnées ont été cultivées sur l'ensemble des milieux mentionnés pendant 48 h, à raison de 48h à 25°C. Les spectres ont été acquis en appliquant le protocole précédemment exposé. Brièvement, à l'issue de cette phase de culture, les échantillons sont déposés sur lame en duplicatas, 1 µL d'acide formique puis 1µL de matrice CHCA sont déposés sur les spots. La souche de calibrant est placée sur les spots appropriés et 1 µL de matrice CHCA

est ajouté pour chaque spot de calibrant. La souche de *Candida glabrata* ATCC MYA-2950 a servi de contrôle qualité après culture sur milieu SDA tandis que la souche d'*Escherichia coli* ATCC 8739 a servi de calibrant après culture sur gélose au sang. Les acquisitions ont par la suite été réalisées grâce au VITEK® MS.

Le critère d'acceptation d'un milieu à l'essai est dit « double ». En effet, afin d'être inclus dans le plan d'expériences, les résultats doivent excéder un seuil de 90% de spectres en concordance avec le milieu de référence ainsi que 90% d'identification correcte. Après traitement des résultats, il est apparu que les milieux DG18, DRBC et PDA ne satisfaisaient à aucun des critères d'acceptation (Tableau 4). Cette inadéquation pourrait résulter de leur composition. Le milieu DG18, conçu pour les organismes xérophiles, contient du dichloran, un antifongique inhibant la croissance des *Mucoraceae* et limitant celle d'autres champignons. De même, le milieu DRBC, enrichi en dichloran, chloramphénicol et rose bengale, peut freiner le développement des espèces fongiques. En revanche, les milieux MEA, YGC et OGA remplissaient les critères d'acceptation avec des taux de concordance et d'identification systématiquement supérieurs à 90%, la majorité excédant 95%. Les milieux jugés compatibles pour l'identification des levures par VITEK MS ont donc été inclus dans le plan d'expérience et utilisés dans la construction de la base de données.

Tableau 4. Synthèse des taux de concordances et d'identifications, comparaison des données acquises pour les milieux tests par rapport au milieu de référence

Nom du milieu	Concordance avec le milieu de référence	Taux d'identification correcte	Compatibilité
DG18	55 %	59 %	Incompatible
DRBC	64 %	68 %	Incompatible
PDA	74 %	68 %	Incompatible
MEA	100 %	100 %	Compatible
YGC	97 %	93 %	Compatible
OGA	97 %	97 %	Compatible

2. Espèces non-retenues lors du développement de la base de données levures-moisissures VITEK® MS KB 3.4

Dans le cadre de l'enrichissement de la base de données spectrales, l'inclusion de plusieurs espèces fongiques sélectionnées n'a pas pu aboutir en raison de l'éviction d'une ou plusieurs souches. En effet, les contrôles qualité réalisés sur certaines souches ont révélé des erreurs d'attribution taxonomique conduisant à l'irrecevabilité des espèces pour manque de diversité génétique. Les espèces ne présentant plus qu'une souche restante sont au nombre de cinq, c'est-à-dire *Aspergillus conicus*, *Candida versatilis*, *Penicillium copticola*, *Saccharomyces pastorianus* et *Trigonopsis cantarelli* (Tableau 5).

Tableau 5. Espèces et numéros des souches des espèces non-admissibles pour l'extension de la base de données

Espèce	Numéro de souche
<i>Aspergillus conicus</i>	<u>UBOCC – A - 115041</u>
<i>Candida versatilis</i>	<u>CBS 1752</u> ^{TS}
<i>Penicillium copticola</i>	<u>EQUASA 81</u>
<i>Saccharomyces pastorianus</i>	<u>CBS 2156</u>
<i>Trigonopsis cantarelli</i>	<u>CBS 4878</u> ^{TS}

* CBS, Westerdijk Fungal Biodiversity Institute culture collection ; EQUASA, Etude En Qualité Alimentaire culture collection ; UBOCC, Université de Bretagne Occidentale Culture Collection

Underlined strain number indicates that strain identification was also confirmed by DNA-sequencing

En conséquence, ces espèces partiellement représentées devront être étendues grâce à l'ajout de nouveaux spectres obtenus à partir d'autres souches. La diversité spectrale liée à ces espèces serait ainsi enrichie afin de remplir les critères d'acceptation (i.e. minimum de deux souches / espèce) permettant par la suite d'intégrer ces espèces à la base de données spectrales.

Les résultats obtenus dans le premier chapitre mettent en évidence la robustesse et la pertinence de la spectrométrie de masse MALDI-TOF pour l'identification des espèces fongiques, y compris celles appartenant à des complexes d'espèces mais également l'intérêt de poursuivre le développement des bases de données spectrales avec des espèces pertinentes. L'utilisation de cette base étendue et aux performances jugées satisfaisantes dans le cadre d'applications concrètes dans des environnements industriels, où la maîtrise des contaminations fongiques constitue un enjeu majeur pour la qualité et la sécurité des produits alimentaires, semble donc d'autant plus pertinente.

Dans ce contexte, le chapitre suivant s'inscrit dans une démarche d'application de cet outil dans un cadre industriel. En effet, ce projet vise à étudier la diversité fongique et les flux de contamination dans une usine de BVP en utilisant à la fois des approches moléculaires et la spectrométrie de masse MALDI-TOF.

Chapitre 2 : Diversité et voies de contamination des moisissures
d'altération de produits de boulangerie en utilisant la spectrométrie de
masse MALDI-TOF et des approches moléculaires

I. Présentation des travaux

Après avoir développé et validé la base de données MALDI-TOF, nous avons souhaité appliquer cette méthode dans un contexte industriel. Pour cela, nous nous sommes appuyés sur la campagne d'échantillonnage d'un projet de recherche en cours au laboratoire, le Projet ANTICIPA, "Jumeau numérique pour la maîtrise des contaminations aéroportées en pâtisserie", porté par une entreprise bretonne de BVP en collaboration avec le LUBEM (Dr. Valérie Vasseur et M. Franck Déniel, avec l'appui technique de Mme Océane Cloarec) et l'Institut National de Recherche pour l'Agriculture, l'alimentation et l'Environnement (INRAE). Ce projet visait à étudier, (i) la diversité fongique dans l'usine et les produits altérés et (ii) le lien entre les contaminations fongiques, notamment aéroportées, les données particulières et la survenue d'altérations fongiques dans les produits avec l'objectif de développer des modèles prédictifs permettant de mieux maîtriser les contaminants fongiques et donc la qualité des produits.

Un échantillonnage de produits finis ainsi qu'une analyse des champignons présents dans l'air et sur les surfaces en contact avec les produits, a été réalisée dans six zones jugées pertinentes dans l'usine, toutes les quatre semaines pendant 42 semaines pendant 1 à 4 jours consécutifs. Les vêtements des opérateurs ont également été échantillonnés. Après conservation des produits finis jusqu'à la fin de leur durée de vie, les moisissures d'altération ont été isolées puis identifiées tandis que des isolats ont également été collectés à partir des échantillons de l'environnement et des vêtements des employés.

L'identification des isolats a été réalisée par spectrométrie de masse MALDI-TOF et par séquençage des gènes d'intérêt taxonomique (actine, β -tubuline, facteur d'élongation, ITS). Par la suite, afin d'identifier les sources de contamination par les espèces fongiques dominantes dans l'usine et sur les produits une étude de diversité intra-spécifique a été menée sur deux espèces en utilisant une approche de génotypage de motifs microsatellites par séquençage haut-débit (SSR-seq). La capacité de discrimination intra-spécifique du MALDI-TOF a été évaluée en se basant sur les résultats du génotypage. L'analyse de la diversité fongique a révélé des

variations dans l'abondance relative des espèces fongiques selon les zones de l'usine et les produits. Les résultats ont montré que les zones avec une qualité d'air non contrôlée, telles que les zones de pré-cuisson présentaient des charges fongiques plus élevées que les zones contrôlées, telles que la sortie du four et les zones de refroidissement. *Cladosporium sphaerospermum* et *Penicillium brevicompactum* étaient parmi les espèces les plus abondantes à la fois dans l'environnement et sur les produits. Le génotypage a montré la co-occurrence d'haplotypes dans l'environnement, notamment les zones post-cuisson et sur les produits altérés. Enfin, l'utilisation de MALDI-TOF MS combinée à l'analyse t-SNE pour le suivi des sources de *C. sphaerospermum* et *P. brevicompactum* a montré une bonne concordance avec les résultats SSR, suggérant un potentiel prometteur pour cette méthode.

II. Article à soumettre dans *International Journal of Food Microbiology*

Rolland, N., Mounier, J., Cadoret, M., Girard, V., Perrin, G., Deniel, F., Tréguer, Cloarec, O., Vasseur, V., Jany, J.-L. Diversity and source-tracking of spoilage molds in bakery products using MALDI-TOF MS and molecular approaches.

Diversity and source-tracking of spoilage molds in bakery products using MALDI-TOF MS and molecular approaches

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Key words : Filamentous fungi – Food spoilage – Strain typing – Spectral database – Industrial context – *Cladosporium sphaerospermum* – *Penicillium brevicompactum*

Abstract

Filamentous fungi are significant contributors to global food losses and waste including bakery products. In the baking industry, mold contamination often occurs at the post-baking stage due to exposure to ambient air. The aim of this study was to investigate fungal diversity and track the environmental sources of spoilage molds in finished products in a French industrial bakery plant, using MALDI-TOF MS and molecular approaches including barcoding and genotyping of microsatellites using next-generation sequencing (SSR-seq). Furthermore, we evaluated MALDI-TOF MS as a rapid tool for the source-tracking of two spoilage molds, i.e., *Cladosporium sphaerospermum* and *Penicillium brevicompactum*. Samples from air, food contact surfaces, work clothes and cakes were collected once every four weeks for 42 weeks on 1-4 consecutive days at 2 times. Results showed that areas with uncontrolled air quality such as the pre-baking area, had higher fungal loads compared to air-controlled areas such as post-baking zones. Products not sprayed with ethanol showed significantly higher mold contamination frequency highlighting the importance of surface treatment in reducing spoilage. *Cladosporium sphaerospermum* and *Penicillium brevicompactum*, known for their xerophilic and airborne dispersal capabilities, were identified as the main spoilers. SSR-seq provided

insights into the genetic diversity of these species and showed that certain haplotypes, particularly originating from post-baking areas, were more frequently found on spoiled cakes suggesting repeated contamination events from persistent sources. Using an unsupervised approach, we showed that there was a good agreement between MALDI-TOF MS and genotyping data analysis at the population level. This study provides new insights into the fungal ecology encountered in the bakery industry.

1. Introduction

Filamentous fungi play a significant role in global food losses and waste. They can colonize a very large diversity of food raw materials and processed foods throughout the food chain, from initial agricultural production to the final consumer (Food and Agriculture Organization of the United Nations (FAO), 2023; Pitt and Hocking, 2009). Globally, it is estimated that fungal spoilage is responsible for 5-10% of food losses and waste (Pitt and Hocking, 2009). In addition to food losses and waste, fungal spoilage also leads to substantial financial losses as well as waste of natural resources (land, water, and greenhouse gas emissions) (Akimowicz and Bucka-Kolendo, 2020; Lücking et al., 2020). Food safety is also a major concern and can be significantly affected by fungal contamination due to potential mycotoxin production (Avery et al., 2019; Filtenborg et al., 1996). Several species within the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* are known to produce toxic secondary metabolites, referred to as mycotoxins, presenting a significant risk for human and animal health (Agence française de sécurité sanitaire des aliments (AFSSA), 2009; Eskola et al., 2020; Smith et al., 2016). Consequently, fungal contamination is a relevant problem in food industries such as dairy and bakery industries (Bernardi et al., 2019; Garnier et al., 2017; Le Lay et al., 2016).

In the context of food spoilage, bakery products present a unique case. These food matrices are generally less prone to microbial spoilage due to the baking process, which eliminates potential contaminants, and their intrinsic properties, such as low water activity, which is incompatible with the growth of most microbial spoilers. However, bakery products are susceptible to post-baking contamination by airborne mold spores present in the environment, including xerotolerant and xerophilic fungi belonging to the *Penicillium*, *Aspergillus*, *Eurotium*, *Wallemia* and *Cladosporium* genera that thrive in dry conditions (Fustier et al., 1998; Morassi

et al., 2018; Moro et al., 2022). Fungal spoilage is usually easy to recognize by the presence of colonies or thalli on the product surface. This fungal growth leads to noticeable changes in product sensorial properties including appearance, taste, flavor, or texture, making the product unfit for consumption (Filtenborg et al., 1996; Nout, 2007). As a result, fungal spoilage shortens the shelf life, limits export possibilities, causes consumer dissatisfaction, and damages the brand's image thereby yielding significant economic losses for both producers and consumers. Therefore, rapidly and accurately identifying those fungal spoilers at the species and possibly intra-species levels and tracking their sources in production facilities is a fundamental aspect for the implementation of corrective actions so that spoilage can be prevented (Bernardi et al., 2019; Filtenborg et al., 1996; Pinheiro et al., 2019; Pitt and Hocking, 2009; Rokas, 2022).

Fungal identification can be performed using various methods, either alone or combined. These techniques include phenotypic approaches, i.e. morphological and biochemical characteristics, and DNA barcoding, which relies on the sequencing of one or more standardized short DNA regions (Antil et al., 2023; Houbraken and Samson, 2017; Quéro, 2018). Due to their tediousness and complexity, these genomic-based approaches, although efficient, tend to be gradually replaced by alternative approaches in routine analysis (Akimowicz and Bucka-Kolendo, 2020b; Antil et al., 2023; Girard et al., 2023; Lücking et al., 2020b; Tran et al., 2015). Among alternative approaches, numerous studies have shown the rapidity and reliability of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) for routine identification of fungi in clinical and industrial microbiology laboratories (Deak et al., 2015; Patel, 2019; Quéro, 2018). More recently, Rolland et al. (2024) expanded the VITEK® MS IVD Knowledge Base V3.4 with 380 new yeast and mold strains belonging to 133 food-relevant species. Levels of 96.55% and 90.48% of correct identification to the species level were achieved for cross and external validation, respectively. In addition, species from five species complexes corresponding to *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Fusarium dimerum*, *Mucor circinelloides* complexes and *Aspergillus* series *Nigri* could be well identified demonstrating the capacity of this technique for differentiation of closely-related and cryptic species within these species complex. While the potential of MALDI-TOF MS to accurately identify fungal species including species complex or cryptic species has been demonstrated (Al-Hatmi et al., 2015; Américo et al., 2020; Quéro et al., 2020), its potential for discrimination of isolates from the same species at the

population and strain levels is still a pending subject. Traditionally, strain typing of fungi is achieved using molecular methods. Among them, two approaches stand out from the others for studying populations and assessing genetic diversity, i.e. single nucleotide polymorphisms sequencing (SNPseq) and microsatellite or simple sequence repeats sequencing (SSRseq) (Tsykun et al., 2017). Noteworthy, simple sequence repeats (SSRs), also named microsatellites, are frequently used markers for fungal sequencing-based genotyping (Li et al., 2009). These genomic-based approaches, although efficient, require expertise, investment at a large scale and their application remains challenging in the case of non-model organisms (Etter et al., 2011; Helyar et al., 2011). In the last decade, the use of MALDI-TOF MS has been explored for typing and serotyping of bacterial pathogens (Sauget et al., 2017; Sloan et al., 2017). For instance, it has been tested on *Escherichia coli* (Lafolie et al., 2015; Nicolas-Chanoine et al., 2014; Siegrist et al., 2007), *Haemophilus influenzae* (Månsson et al., 2015), *Salmonella enterica* (Mangmee et al., 2020), *Staphylococcus aureus* (Sauget et al., 2017; Ueda et al., 2015), *Pseudomonas aeruginosa* (Cabrolier et al., 2015), *Listeria monocytogenes* (Barbuddhe et al., 2008), *Neisseria meningitidis* (Suarez et al., 2015), *Klebsiella pneumoniae* (Berrazeg et al., 2013; Sachse et al., 2014) and *Streptococcus* spp. (Lasch et al., 2014; Nakano et al., 2015; Spinali et al., 2015; Wang et al., 2012). Noteworthy, the effectiveness of MALDI-TOF MS sometimes matched that of molecular methods, but in some cases, an insufficient discriminatory power was reported (Lasch et al., 2014; Spinali et al., 2015). This technique has also been applied for typing of fungi in a food industrial context including yeasts (e.g., *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*) (Lauterbach et al., 2017) and filamentous fungi (e.g., *Penicillium roqueforti*) (Quéro et al., 2020). Rapid, low-cost and accurate identification of species in an industrial context could help optimize prevention and control of fungal spoilage in food. Additionally, going further with population-level or strain level identification could be of great interest not only for source-tracking purpose but also to discriminate toxigenic and non-toxigenic strains, to compare clinical and environmental isolates, to study the domestication process or even to discriminate different strains involved in the fermentation process (Belén Flórez et al., 2007; Nicolas-Chanoine et al., 2014).

In this context, the aim of this study was to investigate fungal diversity in a French industrial bakery plant using MALDI-TOF MS and molecular approaches, with a specific emphasis on tracking the environmental sources of spoilage molds in finished products. Furthermore, we evaluated MALDI-TOF MS as a rapid tool for the source-tracking of two spoilage molds, i.e., *C. sphaerospermum* and *P. brevicompactum*.

2. Material and methods

2.1. Sampling sites, monitoring of mold contamination and product spoilage, and isolation of fungi

Sampling was performed once every four weeks for 42 weeks on 1-4 consecutive days at 2 times (morning and afternoon) during the period March-December 2022 in a French bakery/pastry factory (Brittany, France) manufacturing a wide variety of plain and flavored cakes (a_w ranging between 0.80-0.85, pH 7-7.8). Samples corresponded to air samples (n=1642) and food contact surfaces (n=404) and were collected in six areas of the factory, i.e., changing rooms (changing rooms), cake batter storage area before baking (before baking), oven exit (oven exit), cooling tunnel exit (cooling exit), packaging area before wrapping (before wrapping), intermediate storage space for packaging materials and packed products (intermediate storage) (Supplementary figure 1). Noteworthy, the air quality in the oven exit, cooling tunnel exit and packaging areas was controlled by a positive pressure fresh air system in which air from the outside was filtered through a HEPA filter and pumped into the room. Mold contamination on work clothes (work clothes) surfaces, including trousers and coat/jacket, collected at the end of the working day (n=546) was also evaluated up to week 26 on 1-4 operators from maintenance, production and packaging services. In addition, on each sampling day and period of the day (morning and afternoon), ~10 cakes manufactured on the same day were taken for shelf-life tests.

2.1.1. Air and surface sampling

Air sampling was performed in quadruplicate using a Microflow Alpha ® biocollector (Aquaria, Lacchiarella, Italy) at a 100 L/min flow rate for 1 min onto an in-house malt and yeast extract agar medium (M2Lev, malt extract 20 g/L, yeast extract 3 g/L, penicillin 0.1 g/L, streptomycin 0.1 g/L) followed by incubation for 7 days at 25°C. For food contact surface sampling, a wipe moistened with distilled water supplemented with 10% neutralizing agent solution (Tween 80 3 g/L, Na₂HPO₄ 3.99 g/L, Na₂S₂O₃ 0.78 g/L, soy lecithin 0.3 g/L, L-histidine 0.1 g/L) was applied on a surface area of 90 cm². The wipe was then placed in 50 mL 0.01% Tween 80 and serial dilutions plated on M2Lev as described above. For work clothes sampling, Sabouraud Dextrose Chloramphenicol Neutralizers (SDCN) agar (surface area 28 cm², bioMérieux, Marcy l'Etoile, France) was used, applying a uniform pressure of 500 g on

the surface of the contact plate for 10 s using a Rodac-Weight (International pbi Spa – Novara, Milano, Italy) followed by incubation for 7 days at 25°C. After thalli enumeration and estimation of *Penicillium* and *Cladosporium* spp. counts based on macromorphological characters in log₁₀ thallus forming unit (TFU) (zero values were replaced by 0.001 for calculation purposes), representative isolates were purified from the different samples yielding a collection of 292 isolates which were stored at –80 °C in 20% glycerol until further use.

2.1.2. Product sampling

As mentioned above, on each sampling day and period of the day (morning and afternoon), ~10 cakes (half of them being sprayed with a 80% ethanol solution before packaging) were brought back to the laboratory and subjected to a shelf life test. Cakes were kept in the dark at 19°C until the end of their shelf-life (~2-3 months depending on the product) and examined for visible mold contamination. Prevalence of mold contamination was determined as follows: Frequency = (total positive/total sample) x 100%. Spearman's rank correlation analysis ($P < 0.05$) between fungal counts and frequency of spoiled products with or without ethanol spraying was performed in R 4.2.2 under RStudio using the ‘*Hmisc*’ and ‘*corrplot*’ packages.

Furthermore, spoiled fragments from these cakes (n=109) as well as from spoiled cakes provided by the manufacturer (n=44) were transferred onto M2Lev or M5S5 for *Wallemia* spp. (malt extract 50 g/L, NaCl 50 g/L, agar 15 g/l) for isolation and purification of fungal spoilers yielding a collection of 153 isolates which were stored at –80 °C in 20% glycerol until further use.

2.2. Identification of fungal isolates

Purified isolates were presumptively identified at the genus level through morphological approaches based on the examination of macroscopic characteristics (e.g. color, texture, growth rate) and microscopic structures (asexual and/or sexual spore structures and hyphae). Then, isolates were identified using MALDI-TOF MS and sequencing of genes of taxonomic interest as described below.

2.2.1. Identification by MALDI-TOF MS

For isolate identification and dereplication, MALDI-TOF MS was used. As previously described by Rolland et al. (2024), to assess viability and purity, the cryopreserved isolates were precultured on Sabouraud Dextrose Agar (SDA, bioMérieux, Marcy l'Etoile, France) at 25°C for 2 to 6 d. Molds were then cultivated on Potato Dextrose Agar (PDA, BD, Le Pont de Claix, France) at 25°C for 8 days before spectra acquisition. After incubation, isolates were processed with an extraction protocol using the VITEK® MS mold kit (bioMérieux, Marcy l'Etoile, France). Fungal biomass was scraped from the agar plate surface using a sterile cotton swab moisturized with API Suspension Medium (bioMérieux, Marcy l'Etoile, France) and suspended in 900 µL of 70% ethanol (bioMérieux, Marcy l'Etoile, France). After vortexing for 5 s and a two-minutes centrifugation at 14,000 x g, the supernatant was discarded and the pellet resuspended into 40 µL of 70% formic acid (bioMérieux, Marcy l'Etoile, France). After vortexing for 5 s, 40 µL of acetonitrile (bioMérieux, Marcy l'Etoile, France) were added, vortexed again for 5 s before centrifugation at 14,000 x g for 2 min. The supernatant was deposited in duplicate on a target slide and allowed to dry. Then, it was covered with 1 µL of α -cyano-hydroxycinnamic acid matrix solution (CHCA, bioMérieux, Marcy l'Etoile, France) and left to dry before MALDI-TOF MS analysis.

Spectra were acquired using the VITEK® MS system (bioMérieux, Marcy l'Etoile, France) equipped with the Launchpad version 2.9.5.6 acquisition software as described previously (Rolland et al., 2024). The system was calibrated using fresh cells of *Escherichia coli* ATCC 8739 and a quality control strain, *Aspergillus brasiliensis* ATCC 16404, was included for each reagent kit on each day of spectra acquisition. As described by Girard et al. (2016), spectra were acquired in linear positive extraction mode in a mass range from 2,000 to 20,000 Da using the “Auto-Quality” option. Spectra were generated by the accumulation of 500 laser shots, 100 profiles being acquired from each spot with 5 shots per profile. Raw spectra were then automatically processed through smoothing and peak detection procedures by the acquisition software as described previously (Girard et al., 2016; Rolland et al., 2024). A log base scaling of the peak intensities was applied followed by a L1-normalization. Using the Advanced Spectra Classifier (ASC) algorithm developed by bioMérieux, a predictive model was established to obtain a specific weight bin matrix for each species. The identification was generated by comparing the new spectra to the matrix. According to Girard et al. (2016), the

sum of matching bin weights was calculated to obtain an intermediate score and transformed into multiclass probability estimates using a Gaussian calibration procedure. Spectra were then compared to the VITEK® MS Knowledge Base for identification. A decision algorithm was used to only retain significant matches. A ‘single choice’ was obtained when only one species was retained, while a ‘low discrimination’ result indicated that more than one species was retained. Finally, a ‘no identification’ result was obtained in case of no significant matches or more than four different species retained. Additionally, a single linkage agglomerative clustering algorithm was used to generate dendrograms with the SARAMIS software (bioMérieux, Marcy l’Etoile, France). A threshold similarity of 50% was applied and representative isolates (n=106), including 26 environmental isolates and 153 isolates from spoiled products, were selected for confirmation of species assignment by sequencing.

2.2.2. Identification by sequencing

DNA from the 179 selected isolates was extracted from mycelial plugs using the Mag-Bind Viral DNA/RNA 96 Kit (Omega Bio-tek Inc., Norcross, GA, USA) according to the manufacturer’s instructions on the Kingfisher Duo Prime purification system (Thermo Fisher Scientific, Waltham, MA, USA). One gene was amplified and sequenced for each isolate depending on their genera, i.e. partial actin gene using primers ACT-512F (5'-ATGTGCAAGGCCGGTTTCGC-3') and ACT-783R (5'-TACGAGTCCTTCTGGCCCAT-3') (Carbone and Kohn, 1999) for *Cladosporium* spp., partial β - tubulin gene using primers Bt2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2B (5'-ACCCTCAGTG TAGTGACCCTTGGC-3') (Glass and Donaldson, 1995) for *Penicillium* spp. and *Aspergillus* spp. isolates, partial elongation factor alpha-1 using primers EF1F (5'-ATGGGTAAGGAGGACAAGACTCA-3') and EF1R (TGGAGATACCAGCCTCGAAC-3') (O'Donnell et al., 1998) for *Fusarium* spp. and the internal transcribed spacer (ITS) region using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990) for other genera. Sequencing was performed at Eurofins Genomics (Edersberg, Germany). Sequences were assembled into contigs using the Geneious Prime software v2024.0.5 (www.geneious.com) and were compared to the GenBank database using the “Basic Local Alignment Search Tool” (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences have been deposited in Genbank under the accession number XXXX-XXXX.

2.2.3 Fungal Species Diversity Analysis

The relative abundance of each fungal species was calculated both for the environmental samples (from the different factory areas) and for the cakes. For each sample, the relative abundance was defined as the proportion of occurrences of a given species relative to the total number of isolates in that particular area or cake sample. This calculation was performed as follows:

$$\text{Relative abundance of species } X = \frac{\text{Number of isolates of species } X}{\text{Total number of isolates in the sample}}$$

This method was applied separately to both the bakery areas and cake samples, and the results were expressed as percentages for each species.

To visually represent the relative abundances of the most frequently occurring fungal species, bubble plots were generated using the ggplot2 package version 3.5.1 in R. To assess the similarities and differences in fungal community composition between the various factory areas, a Non-metric Multidimensional Scaling (NMDS) analysis was performed. The NMDS was conducted using the Bray-Curtis dissimilarity matrix, which is appropriate for ecological community data. The analysis was performed with the metaMDS function from the vegan package version 2.6.8 in R, using a maximum of 100 iterations to ensure convergence. The NMDS stress value was used to evaluate the goodness-of-fit of the representation in two dimensions, with a stress value below 0.1 considered as a good fit.

2.3. Source-tracking of main spoilage fungi

2.3.1 SSR-seq analysis

SSR-seq analysis was performed on monosporal cultures of *C. sphaerospermum* (n=82 including 43 from environmental sources and 39 from spoiled products) and *P. brevicompactum* (n=35 including 32 from environmental sources and 3 from spoiled products). Nine *C. sphaerospermum* (UBOCC-A-113067, UBOCC-A-101107, UBOCC-A-112116, UBOCC-A-113031, UBOCC-A-118061, UBOCC-A-108054, UBOCC-A-117126, UBOCC-A-113079 and UBOCC-A-113080) and 13 *P. brevicompactum* (UBOCC-A-110202, UBOCC-A-111022,

UBOCC-A-110008, UBOCC-A-110065, UBOCC-A-111142, UBOCC-A-112048, UBOCC-A-112078, UBOCC-A-112087, UBOCC-A-112164, UBOCC-A-112165, UBOCC-A-118164, UBOCC-A-114102 and UBOCC-A-114103) isolates from the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) were also included.

To obtain monosporal isolates, isolates were grown on PDA (Thermo Fisher Scientific, Le Pont de Claix, France) at 25°C for 3 to 4 d. Spore suspensions were prepared by adding 500 µL of 0.01% Tween 80 on the plate surface (3 drops of Tween® 80 for 1 L of solution; Sigma Aldrich) and then gently scraping the surface with a sterile rake shaped spreader. After spore enumeration using a Malassez cell, ten-fold serial dilutions (up to 10⁻²) in 0.01% Tween 80 were prepared. Then, 100 µL of 10⁻¹ and 10⁻² dilutions were spread onto PDA plates containing 16 mL of agar medium and incubated at 25°C for 24 to 48 h. Using a binocular microscope, germinating spores were then picked up using a Pasteur pipette and then placed onto a new PDA plate and incubated at 25°C for 7 d. Monosporal isolates were stored at -80 °C in 20% glycerol until further use.

DNA extraction from the monosporal isolates was performed as described above. DNA concentration was estimated using a NanoDrop ® 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to a final concentration between 10 and 15 ng/mL.

SSR-sequencing, which included primer design, microsatellite sequencing and bioinformatic analysis, was performed at the ‘Plateforme Génome Transcriptome de Bordeaux’ (Univ. Bordeaux, INRAE, BIOGECO, F-33610 Cestas, France). Briefly, as described in Lepais et al. (2020), the QDD v 3.1 pipeline (Megléczy et al., 2014) was used based on NCBI genomes of *C. sphaerospermum* (accession number: GCA_019191205.1) and *P. brevicompactum* (accession number: GCA_028827555.1) to detect microsatellites and design specific primer pairs. Among the designed primers, about 60 were retained based on amplification, higher probability of polymorphic loci and quality criteria. A three-round multiplex Polymerase Chain Reaction (PCR) was applied to simultaneously amplify all loci. As described by Lepais et al. (2020), during the third round of PCR, Illumina sequencing adaptors and barcodes were added to assign each sequence to an individual. The reads were then sequenced using a P1 flow cell of an Illumina NextSeq 2000 sequencer in a paired-end 2 x 150 bp configuration (Lepais et al.,

2020). Finally, after quality control using the FDSTools v1.1.1 pipeline (Hoogenboom et al., 2017), we ultimately filtered loci and individuals with a missing data rate greater than 10%. The missing data could be attributed to either amplification failure or the presence of null alleles, but it was impossible to definitively determine the cause. A total of 36 SSR loci were retained and used for the analysis of 64 *C. sphaerospermum* isolates, while 13 SSR loci were retained and used for the analysis of 28 *P. brevicompactum* isolates.

Both *C. sphaerospermum* and *P. brevicompactum* allele datasets were converted into genind objects using the d2fgenind function from the adegenet package version 2.1.10 (Jombart, 2008), specifying a ploidy of 1 due to the haploid nature of both species. Allelic diversity and haplotype distribution were estimated using the R package poppr (Kamvar et al., 2014). The poppr v2.9.4 R package (Kamvar et al. 2015) was employed to evaluate allelic diversity and haplotype distribution by estimating the number of multilocus genotypes (MLG), the expected number of MLGs adjusted for sample size (eMLG), Shannon-Wiener index of MLG index (H; Shannon 1948), Nei's expected heterozygosity (Nei, 1978) and the global linkage disequilibrium, estimated using the index of association and its standardized form (Ia; Brown et al. 1980, and rbarD; Agapow & Burt 2001). To further examine the genetic linkage between individual pairs of loci and identify the specific pairs of loci that exhibited significant linkage disequilibrium, genetic linkage disequilibrium among loci was assessed using the ia() and pair.ia() functions from the poppr package in R.

In order to infer population structure in both *C. sphaerospermum* and *P. brevicompactum*, we employed the individual-based Bayesian clustering methods implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) through the Galaxy platform (<https://usegalaxy>). STRUCTURE makes use of Markov Chain Monte Carlo (MCMC) simulations to infer the proportion of ancestry of genotypes from K distinct clusters. The underlying algorithms attempt to minimize deviations from Hardy–Weinberg and linkage disequilibria. Ten independent analyses were carried out for each number of clusters, from $K = 2$ to $K = 10$, using admixture models and 500,000 MCMC iterations, after a burn in of 50,000 steps. To determine the most robust population structure, we applied the Evanno method (Evanno et al., 2005) to both datasets using the Cluster Markov Packager Across K (CLUMPAK, <https://tau.evolveq.net/clumpak/>), which processed the STRUCTURE results and identified the optimal K value and corresponding clustering solutions for each dataset.

We used the R package *ade4* version 1.7–22 (Bougeard and Dray, 2018; Chessel et al., 2004; Dray and Dufour, 2007; Thioulouse et al., 2018) to perform Principal Coordinate Analysis (PCoA). For both the *C. sphaerospermum* and *P. brevicompactum* datasets, the genetic data were formatted into a *genind* object, and a Euclidean distance matrix was calculated. PCoA was then conducted using the *dudi.pco* function. To assess genetic differentiation among the *C. sphaerospermum* and *P. brevicompactum* populations, we calculated pairwise F_{ST} values using both a complete set of SSR markers and a reduced set where SSR markers showing significant linkage disequilibrium ($r_{barD} \geq 0.8$) were excluded using the method of Weir and Cockerham (1984). The reduced set was used because F_{ST} calculations are particularly sensitive to linkage disequilibrium. SSR data were analyzed using the R package *poppr* for data handling, and F_{ST} values were computed both on the complete dataset and the reduced datasets with the *pairwise.WCfst* function from the *hierfstat* package version 0.5-11 (Goudet, 2005). The populations were predefined based on prior STRUCTURE analysis.

To investigate the spatial distribution and co-occurrence of *C. sphaerospermum* and *P. brevicompactum* haplotypes within the bakery environment, we performed a co-occurrence network analysis using custom R scripts. The co-occurrence of haplotypes across different bakery areas was quantified by constructing a contingency table, which was subsequently converted into a network graph using the *igraph* version 2.0.3 R package (Csardi and Nepusz, 2005).

2.3.2. Differentiation of *Cladosporium sphaerospermum* and *Penicillium brevicompactum* isolates using MALDI-TOF MS

Monosporal isolates were analyzed using MALDI-TOF MS, following the same procedure as described above.

Prior to the assessment of MALDI-TOF MS discriminating power at the intra-species level, a recalibration procedure was applied to MS data in the absence of a reference mass. The goal was to identify a set of reference masses from the dataset itself, to be used for subsequent

alignment of spectra. The recalibration process involved the identification of common masses and the selection of reference masses. For the identification of common masses, we first searched for spectra with a significant number of common peaks. Specifically, spectra containing at least 10 peaks that were "close" to one another, within a tolerance of 650 ppm, were considered. From this subset, the median mass was calculated for each cluster of peaks, serving as the reference mass. After applying this method to our dataset, 13 common masses were identified across 80% and 85% of the spectra for *C. sphaerospermum* and *P. brevicompactum*, respectively. These masses were 5126.785, 5139.47, 5695.8, 6475.35, 6821.08, 6940.21, 7597.65, 7617.95, 7991.92, 8016.995, 8244.97, 8410.3, and 8475.99 Da for *C. sphaerospermum*, and 3656.395, 3785.44, 4636.82, 4788.98, 4877.56, 4917.41, 4933.18, 4956.68, 5236.5, 5415.28, 6562.78, 6946.88, 9367.17 Da for *P. brevicompactum*. These reference masses were then used to recalibrate the spectra to correct for any mass deviations by applying a linear model across the dataset. This method ensures that the recalibrated spectra are aligned in a consistent manner, improving the reliability of downstream analyses.

After recalibration, a non-supervised approach based on at-Stochastic Neighbor Embedding (t-SNE) method was used to visualize the distance between MALDI-TOF MS spectra for each species as previously described (Rolland et al. 2024) in Plotly.js version 2.27.0 (Hinton and Roweis, 2002; Maaten and Hinton, 2008). The t-SNE method focuses on preserving local structure, making it particularly effective at revealing clusters in data (Maaten and Hinton, 2008).

3. Results

3.1. Monitoring of environmental mold contamination and product spoilage

An extensive monitoring of environmental mold contamination was conducted in the present study including air, surfaces and on work clothes of operators (Figure 1). As shown in Figure 1A, airborne mold counts did not exceed $\sim 3 \log_{10}$ TFU/m³ in the factory, independently of sampled areas. In changing rooms and before baking areas, total fungal counts were quite stable during the studied period, *i.e.*, ~ 2.5 - $3 \log_{10}$ TFU/m³, while those of *Cladosporium* and *Penicillium* spp. counts were 1-2 \log_{10} lower than that of total fungal counts. As expected, total fungi, *Cladosporium* and *Penicillium* spp. counts were 1-2 \log_{10} lower (mean counts ranging from 1-2 \log_{10} TFU/m³ and < 1 -1 \log_{10} TFU/m³, respectively) in the oven exit area, cooling

exit area and packaging area which were pressurized and equipped with air filtration systems while counts in the intermediate storage area for packaging and packed products were slightly higher ($\sim 1.5\text{-}2 \log_{10} \text{TFU}/\text{m}^3$) than the latter areas. Noteworthy, as reflected by the high standard deviation observed in total fungal counts, there were important intra-week (Figure 1A) and intra-day variations (data not shown) in these zones. Similar observations were also made concerning *Penicillium* and *Cladosporium* spp. counts (data not shown). As shown in Figure 1B, surface mold counts ranged between 2 to 5 $\log_{10} \text{TFU}/100 \text{cm}^2$. Interestingly, the trend observed for airborne fungal spores was also observed for surfaces but to a lower extent. Indeed, surface mold counts were generally higher in the changing room and area before baking than in the other zones but differences in mold counts were much lower than those observed for the air with a $\sim 0.5\text{-}1 \log_{10} \text{TFU}/100 \text{cm}^2$ difference. Once again, important intra-week (Figure 1B) and intra-day variations (data shown) were also observed. As shown in Figure 1C, work clothes of operators, which were sampled at the end of the working day, had total fungal counts ranging from 1.5 to 3 $\log_{10} \text{TFU}/100 \text{cm}^2$ with quite similar counts for coat/jacket and trousers. Estimated mean *Penicillium* and *Cladosporium* spp. counts were generally below 1 $\log_{10} \text{TFU}/\text{cm}^2$.

In Figure 1D is shown the frequency of spoiled products (i.e., visible molds on the product surface) at the end of the shelf life for products manufactured during the sampling campaign. Overall, the frequency of spoiled products was very low for products sprayed with ethanol ranging from 0 to 8% while that for products not sprayed with ethanol could reach a weekly mean frequency of up to 35-45% on week 12, 16, 26 and 38 while it ranged between 0 to 18% for other sampling weeks. It is also worth mentioning that within the same week, high variations in the number of spoiled products without ethanol spraying could be observed.

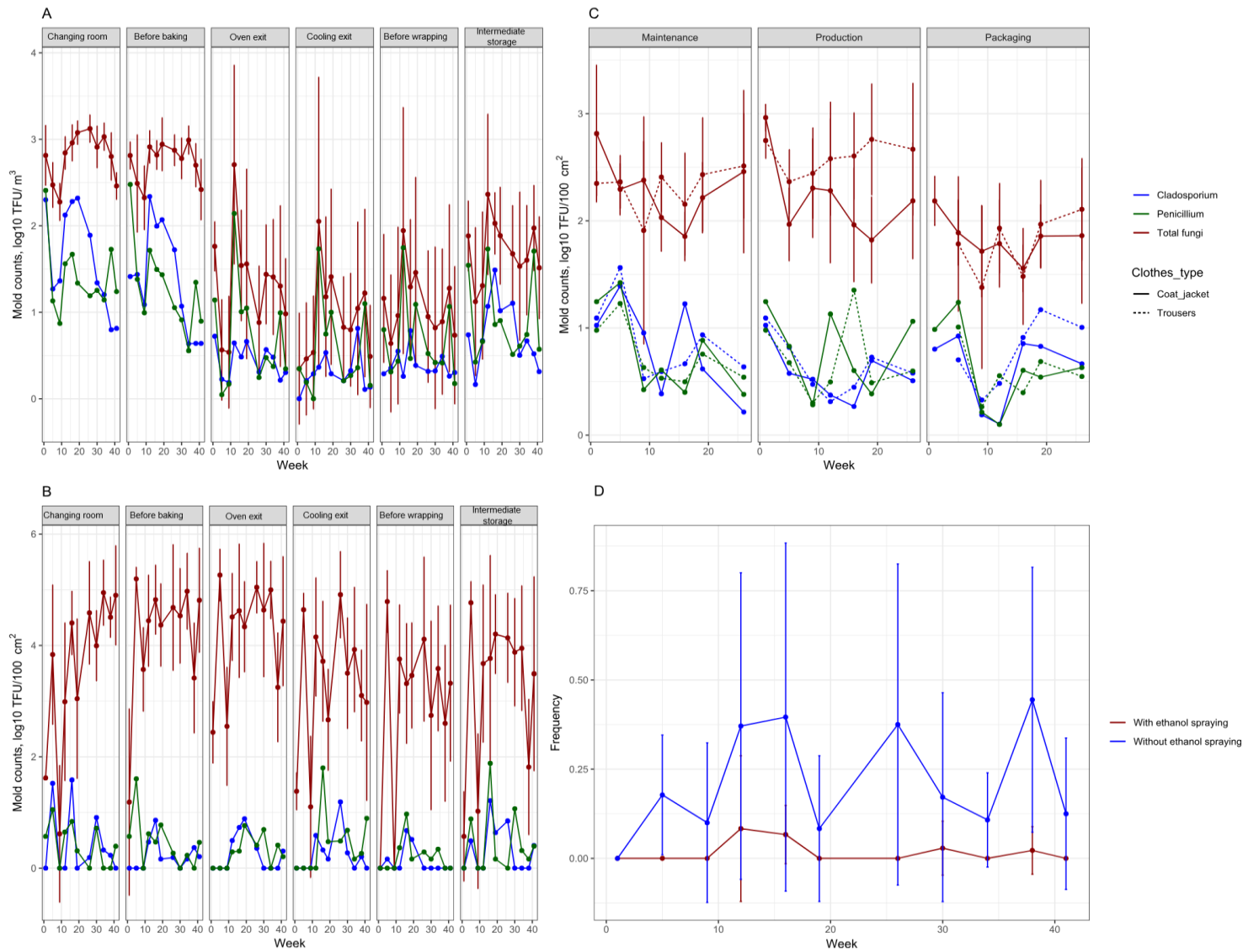


Figure 1. Total fungal counts, estimated *Cladosporium* and *Penicillium* spp. counts in factory air (A) and surfaces (B) and on operator clothes (C). (D) Frequency of spoiled products with or without ethanol spraying.

We then looked for correlations between airborne fungal counts at the intra-day level (morning and afternoon) and frequency of spoiled products at the end of shelf-life that were manufactured at the same time-period. As expected, within the same area, significant correlations ($P < 0.05$) were found between total fungal counts and those of *Cladosporium* and *Penicillium* spp. (Supplementary Figure 2). In addition, significant but moderate correlations ($P < 0.05$, r ranging between 0.3-0.5) were also found between fungal counts from different areas. This was especially true for fungal counts observed in the oven exit area, cooling exit area, packaging area and intermediate storage area for packaging and packed products indicating that temporal variations of fungal counts in these areas were quite similar. Interestingly, significant but weak correlations were also observed between the spoilage frequency of pound cakes sprayed with ethanol and counts of total fungi ($r = 0.3$) and *Penicillium* spp. ($r = 0.35$) in the cooling exit area as well as with those observed in the packaging area ($r = 0.34$ for total fungal counts and $r = 0.37$ for *Penicillium* spp. counts) (Supplementary Figure 2). Concerning the spoilage frequency of pound cakes not sprayed with ethanol, significant but weak correlations were only found with total fungal ($r = 0.28$) and *Penicillium* spp. ($r = 0.31$) counts in the cooling exit area (Supplementary Figure 2).

3.2. Fungal species diversity

3.2.1. Fungal species diversity in spoiled products

A total of 153 isolates were obtained from spoiled cakes of which 25 and 128 were obtained from cakes manufactured with and without ethanol spraying, respectively, and identified using MALDI-TOF MS and/or sequencing. Independently of the cake treatment, they belonged to six genera and 31 taxa with *Cladosporium* ($n=68$, 6 taxa), *Penicillium* ($n=36$, 14 taxa), *Wallemia* ($n=28$, 1 taxa) and *Aspergillus* ($n=15$, 8 taxa) as the most frequently and abundantly isolated genera (supplementary Table 1). Noteworthy, A chi-square test of independence showed that there was not any significant relationship ($P = 0.57$) between cake treatment and the number of isolates from each genus, indicating that there was not any effect of surface treatment on spoiler diversity at the genus level. However, fungal spoiler species diversity was much more reduced on cakes with ethanol spraying as only 12 taxa (*vs* 36 taxa) were isolated from such products, suggesting that ethanol treatment, besides decreasing the odds of fungal spoilage, provided a hurdle effect towards numerous species (supplementary Table 1).

In Figure 2 are shown the relative abundance of the top 10 taxa isolated from spoiled products. *C. sphaerospermum* was the most prevalent species (n=63) followed by members of *Wallemia sebi* species complex (n=28), *P. brevicompactum* (n=11) and members of the *Aspergillus* section *Aspergillus* (formerly *Eurotium* spp., n=11), all of which being isolated throughout the sampling campaign (data not shown). Noteworthy, with the exception of *P. brevicompactum*, these taxa were isolated from cakes with or without ethanol spraying. Other less frequently isolated species included *Penicillium* spp. (i.e., *Penicillium chrysogenum*, *Penicillium crustosum* and *Penicillium corylophilum*), *Aureobasidium melanogenum* as well as members of the *Aspergillus* section *Flavi*.

3.2.2. Fungal species diversity within the factory environment

A total of 235 isolates were collected from air and surfaces within the bakery, as well as from working clothes, and identified using MALDI-TOF MS and/or sequencing. These isolates belonged to nine genera, with up to 42 distinct taxa identified (Supplementary Table 2). *C. sphaerospermum* (125 isolates) and *Penicillium* spp. (96 isolates) were the most frequently and abundantly isolated taxa (Supplementary Table 2). In particular, *C. sphaerospermum*, species from the *Cladosporium cladosporioides* complex, and *P. brevicompactum* were the three most prominent taxa. *C. sphaerospermum* and species from the *C. cladosporioides* complex were consistently found across all sampled areas of the bakery (Figure 2). Other species were not found in every area but were still widespread. For example, *P. brevicompactum*, the second most abundant species in the bakery, was not detected in the pre-baking zone (mixing area). Some species showed a more specific distribution, such as species from the *Aspergillus* section *Flavi*, which were found exclusively in the pre-baking zone, or *P. corylophilum*, which was isolated only in the intermediate storage area. Among the species abundantly present on the cakes, it is noteworthy that *Wallemia* spp. and *A. melanogenum* were not identified in the factory environment, while only few isolates from *Aspergillus* section *Aspergillus* (former *Eurotium* sp.) were identified.

The NMDS results (Figure 3) allowed us to visualize the similarities and differences in fungal composition across the factory areas.

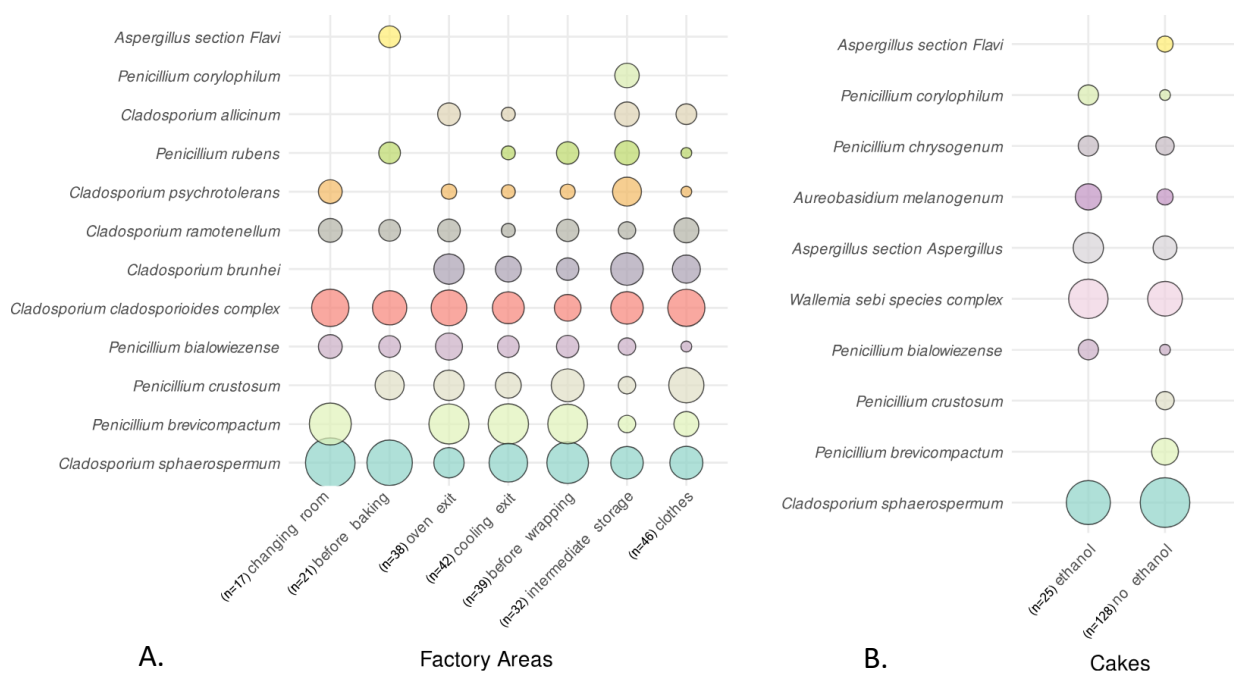


Figure 2. Relative abundance of fungal species across factory areas and cakes (treated and untreated). (A) Bubble plot representing the relative abundance of the top 10 most abundant fungal species identified in different areas of the bakery, including changing room, before baking, oven exit, cooling exit, before wrapping, intermediate storage, and clothes. Each bubble's size corresponds to the relative abundance of a species in a specific area, calculated as the number of occurrences of a species divided by the total number of isolates in that area. Larger bubbles indicate higher relative abundance. The color of each bubble corresponds to a specific fungal species. (B) Bubble plot representing the relative abundance of fungal species identified on cakes treated with an ethanol spray (ethanol, n=25) and untreated cakes (no_ethanol, n=128). The species are aligned with those present in the factory areas when applicable. Bubble size reflects the relative abundance of a species on the cakes, with larger bubbles indicating higher abundance. The total number of isolates (n) for each area or type of cake is indicated next to the area labels.

The NMDS stress value of 0.046 indicates a strong representation of the data in two dimensions, providing a reliable approximation of dissimilarities between samples. The before baking and intermediate storage areas were notably distant from the other zones, indicating a distinct fungal composition in these areas. In contrast, fungal composition in other areas such as the oven exit and before wrapping areas as well as that of clothes were more closely clustered, suggesting a similar fungal community in these sampling sites.

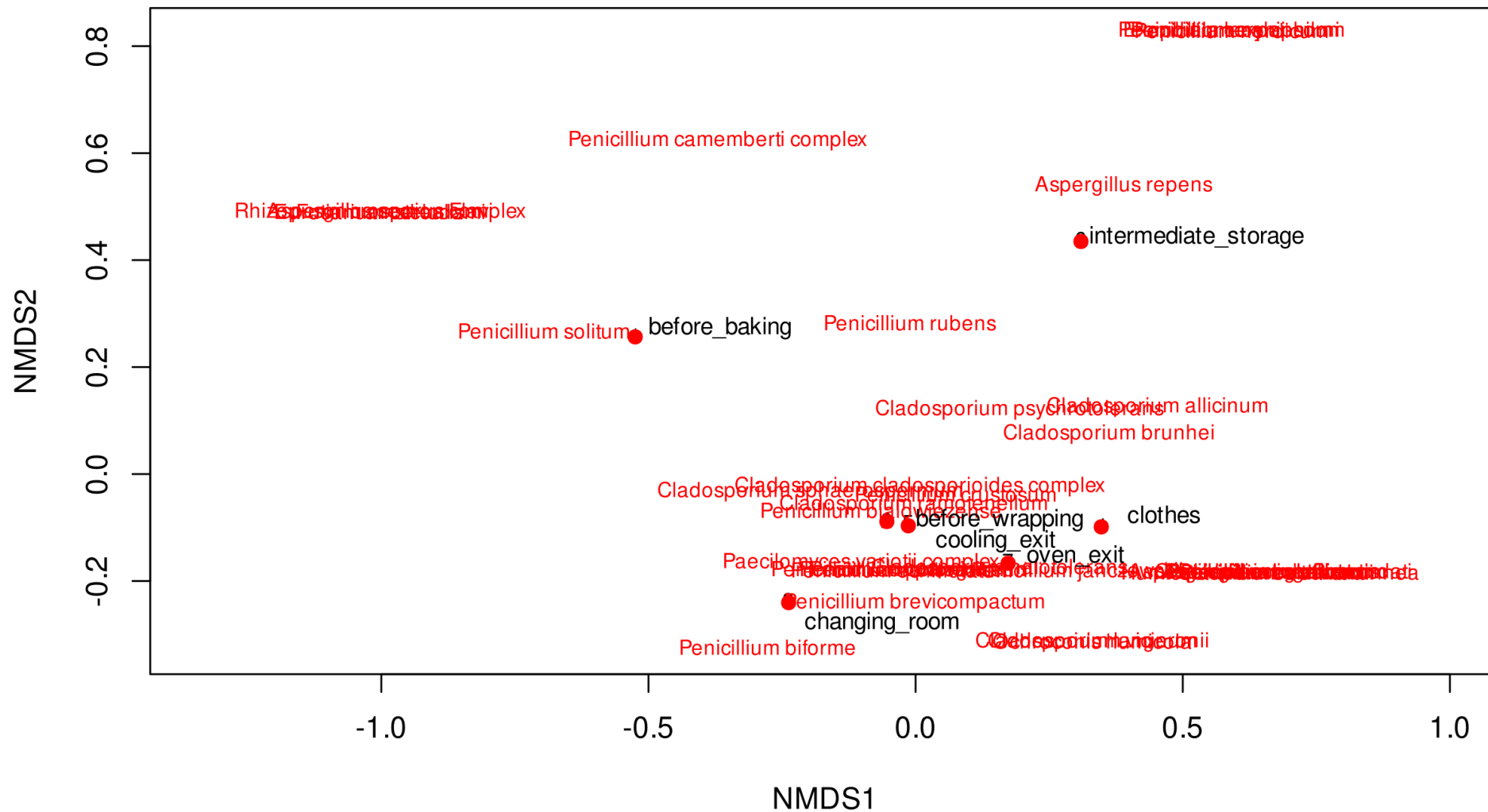


Figure 3. Non-metric Multidimensional Scaling (NMDS) analysis of fungal species composition in different factory areas based on relative abundances (NMDS stress value = 0.046). Each red point represents a sample (i.e., factory areas, work clothes), and the distance between points reflects the degree of similarity or dissimilarity in fungal composition.

3.3. Source-tracking

3.3.1. SSR analysis

Sixty-four *C. sphaerospermum* isolates sampled from the bakery were analyzed using 36 SSR markers. Across the 36 markers, the number of alleles per locus ranged from 2 to 8 (Table 1), with an average of 4.22 alleles per locus (Table 2). A total of 22 distinct multilocus genotypes (MLGs) or haplotypes were identified among the 64 isolates (Supplementary Table 3). The eMLG ($eMLG = 6.37$), average expected heterozygosity ($H_{exp} = 0.40$) and the Shannon's diversity index ($H = 2.39$) indicated a moderate level of genetic diversity within the overall population (Table 2).

Table 1. Summary of genetic diversity metrics for each SSR locus analyzed in the *Cladosporium sphaerospermum* isolate. The table includes the SSR marker name (asterisks indicate SSR markers retained after excluding those with significant linkage disequilibrium, $r_{barD} \geq 0.8$), Simpson's Diversity Index (1-D), Nei's Expected Heterozygosity (H_{exp}), and Evenness.

SSR marker	Repeat	Allele number	1-D	Hexp	Evenness
CS_SSR01*	(AGGC) ₂₋₁₀	4.00	0.33	0.33	0.50
CS_SSR02	(GAC) ₂₋₉ GAA(GAC) ₀₋₉	5.00	0.45	0.46	0.55
CS_SSR03	(CTA) ₀₋₆	2.00	0.30	0.31	0.71
CS_SSR04*	(ACT) ₃₋₇	5.00	0.61	0.62	0.72
CS_SSR06*	(CAG) ₄₋₁₀	5.00	0.39	0.40	0.51
CS_SSR07	(GCA) ₃₋₁₁	4.00	0.31	0.31	0.51
CS_SSR08	(AGC) ₁₋₁₁ N(AGC) ₀₋₃	4.00	0.33	0.33	0.50
CS_SSR09	(CAG) ₃₋₁₂	5.00	0.33	0.33	0.46
CS_SSR10*	(CTC) ₁₋₄ (CTG) ₃₋₁₄	7.00	0.53	0.54	0.51
CS_SSR11*	(CTG) ₅₋₁₀	4.00	0.34	0.35	0.50
CS_SSR12	(CAG) ₃₋₁₁	4.00	0.42	0.42	0.55
CS_SSR13	(TGC) ₁₋₄	4.00	0.34	0.34	0.57

SSR marker	Repeat	Allele number	1-D	Hexp	Evenness
CS_SSR14*	(GGA) ₂₋₉	5.00	0.40	0.40	0.49
CS_SSR16*	(TTG) ₁₋₁₃	5.00	0.35	0.36	0.48
CS_SSR17	(ACA) ₁₋₁₁	6.00	0.66	0.67	0.66
CS_SSR18*	(CTT) ₃₋₁₅	3.00	0.32	0.33	0.56
CS_SSR22*	(TCT) ₂₋₁₈	4.00	0.33	0.33	0.50
CS_SSR24*	(GAT) ₂₋₁₁	5.00	0.65	0.66	0.76
CS_SSR25*	(TCA) ₄₋₈	4.00	0.59	0.60	0.73
CS_SSR27	(CAT) ₂₋₁₃	4.00	0.32	0.33	0.51
CS_SSR28	(GAT) ₁₋₉	3.00	0.32	0.33	0.56
CS_SSR29*	(GAT) ₁₋₉	4.00	0.53	0.54	0.75
CS_SSR33	(CA) ₂₋₁₈	3.00	0.16	0.17	0.48
CS_SSR34	(CA) ₁₋₂₁	4.00	0.31	0.31	0.51
CS_SSR35*	(AC) ₁₋₁₈	4.00	0.56	0.57	0.74
CS_SSR36	(CA) ₂₋₁₁	4.00	0.33	0.33	0.50
CS_SSR38	(CA) ₃₋₉	4.00	0.33	0.33	0.50
CS_SSR42*	(CT) ₂₋₇	4.00	0.50	0.51	0.65
CS_SSR46*	(GA) ₃₋₂₃	7.00	0.38	0.38	0.42
CS_SSR47*	(GA) ₃₋₉	4.00	0.33	0.33	0.50
CS_SSR52	(TC) ₃₋₁₁	5.00	0.35	0.36	0.48
CS_SSR53*	(CT) ₅₋₇	4.00	0.33	0.33	0.50
CS_SSR56*	(CT) ₆₋₁₃	3.00	0.60	0.60	0.86
CS_SSR57*	(CT) ₄₋₁₀	4.00	0.33	0.33	0.50
CS_SSR58	(CT) ₁₋₉	3.00	0.32	0.33	0.56
CS_SSR59*	(CT) ₁₋₇	3.00	0.32	0.33	0.56
mean	-	4.22	0.40	0.40	0.57

Table 2. Summary of genetic diversity and linkage disequilibrium metrics across different *Cladosporium sphaerospermum* populations. The table includes the population identifier (Population), the number of isolates for each population or overall (Isolate Number), the number of distinct multilocus genotypes identified (MLG), the expected number of MLGs accounting for sampling effort (eMLG), the standard error of the eMLG estimate (SE), Shannon-Wiener diversity index (H), Nei's expected heterozygosity (Hexp), index of association (Ia), and standardized index of association (rbarD).

Population	Isolate number	MLG	eMLG	SE	H	Hexp	Ia	rbarD
1	52	12	5.19	1.18	1.83	0.13	3.62	0.23
2	5	4	4.00	0	1.33	0	-0.11	-0.06
3	5	4	4.00	0	1.33	0	0.13	0.14
4	2	2	2.00	0	0.69	0	NA	NA
Overall	64	22	6.37	1.32	2.39	0.40	25.23	0.72

We investigated the population structure of the sampled isolates using the STRUCTURE program. STRUCTURE identified well-defined clusters at K values up to four (Supplementary Figure 3), with no admixture between clusters (Figure 3.B), suggesting the presence of four genetically differentiated clusters among the *C. sphaerospermum* isolates. While these clusters may correspond to distinct populations, the localized nature of our sampling limits broader conclusions about the species population structure. The deltaK value confirmed that this four-cluster model was the strongest structure in the dataset (Figure 3.C). PCoA was in accordance with the existence of these four well separated clusters, with the results showing separation between them (Figure 3.A). Cluster 1, although the largest, exhibited a relatively low genetic diversity with only 12 multilocus genotypes (MLGs) identified among 52 individuals (Table 2). Cluster 2, with 5 isolates, and Cluster 3, also with 5 isolates, both showed moderate genetic diversity, each harboring 4 MLGs (Table 2). Cluster 4, which consisted of only 2 isolates, had 2 MLGs, but the sample size was too small to draw meaningful conclusions (Table 2).

The calculation of F_{ST} values, regardless of whether we used the full set of 28 SSR markers or the reduced dataset of 8 SSR markers, also supported the population structure described above (the reduced dataset was used because F_{ST} calculations are highly sensitive to linkage disequilibrium). All F_{ST} values were relatively high, within the 0.29 – 0.40 range (Table 3), indicating significant genetic differentiation between the clusters.

Table 3. Pairwise F_{ST} values representing the genetic differentiation between the four populations in *Cladosporium sphaerospermum*, identified using 36 SSR markers. Values in parentheses represent F_{ST} values calculated using a reduced dataset of 20 SSR markers, where markers showing significant linkage disequilibrium ($r_{barD} \geq 0.8$) were excluded. F_{ST} values range from 0 to 1, with higher values indicating greater genetic differentiation. "-" denotes comparisons within the same population, where F_{ST} is not applicable.

	Population 1	Population 2	Population 3	Population 4
Population 1	-	0.37 (0.13)	0.37 (0.35)	0.39(0.48)
Population 2	0.37 (0.13)	-	0.31 (0.26)	0.32 (0.36)
Population 3	0.37 (0.35)	0.31 (0.26)	-	0.29 (0.17)
Population 4	0.39 (0.48)	0.32 (0.36)	0.29 (0.17)	-

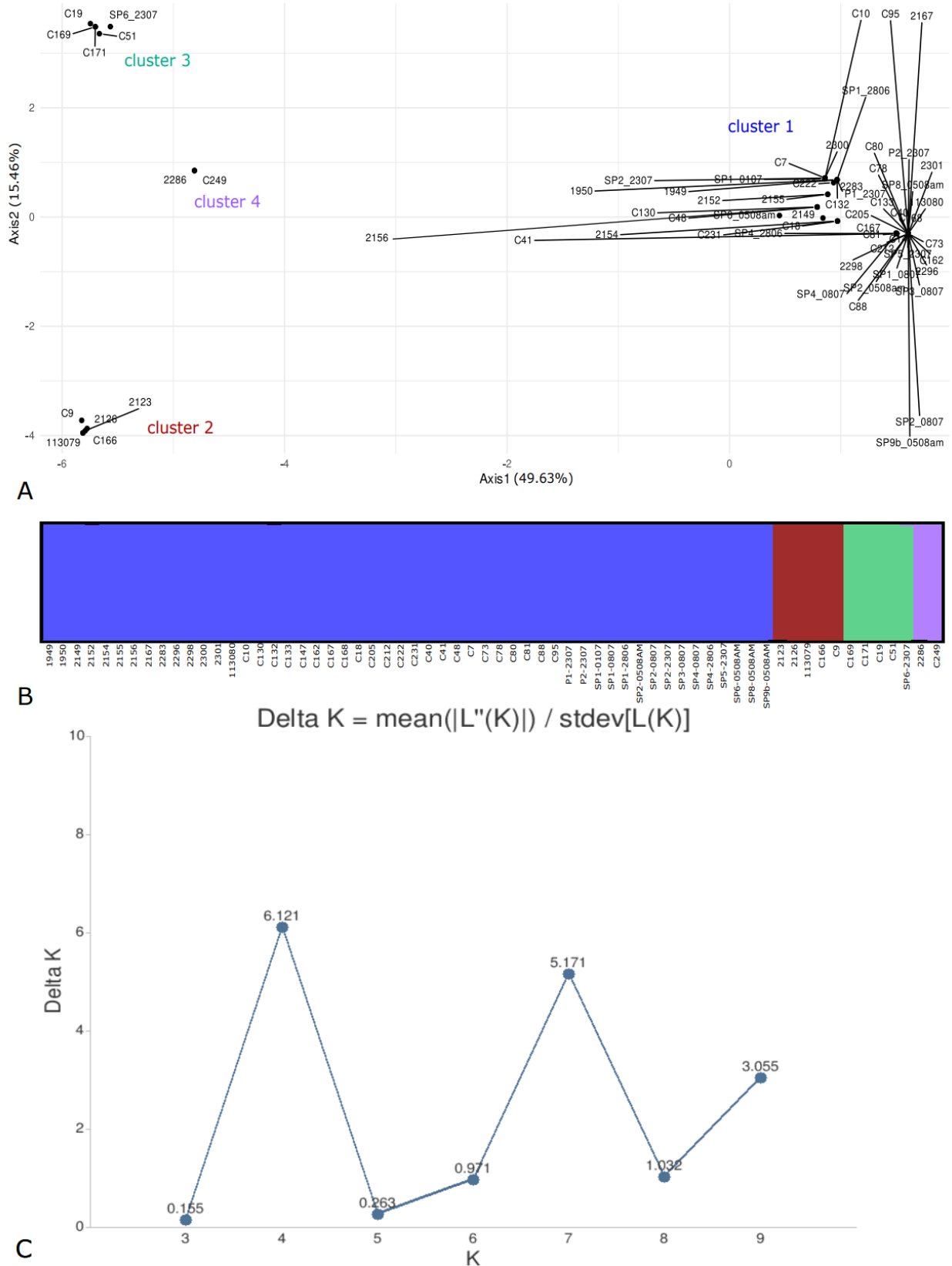


Figure 3. Principal Correspondence Analysis performed using R on the 64 *C. sphaerospermum* isolates (A). Detection of the uppermost level of structure of the *C. sphaerospermum* dataset. Delta K data obtained using Evanno method and inferred by STRUCTURE (B). Population structure of *C. sphaerospermum* (inferred by STRUCTURE for K = 4) (C).

To explore the spatial distribution and co-occurrence of *C. sphaerospermum* haplotypes within the bakery environment, we performed a co-occurrence network analysis (Figure 4). The analysis revealed that haplotypes from different genetic clusters co-occur within specific bakery areas, with evidence of cluster-specific clustering in some areas. The cake samples served as a central node in the network, with numerous and thick edges connecting them to a wide range of haplotypes. Contaminants sampled from cakes appear to originate from diverse genetic clusters and multiple sources within the bakery. The cooling tunnel exit (cooling_exit in Figure 4) and the Packaging area before wrapping (before_wrapping in Figure 4) areas show the strongest connections to the cake node, particularly through haplotype 23 from the genetic cluster 1. Conversely, areas such as oven exit and changing room exhibit weaker or fewer connections to the cake node, suggesting that these areas are less significant contributors to cake contamination, possibly due to lower microbial load or effective containment practices in these zones.

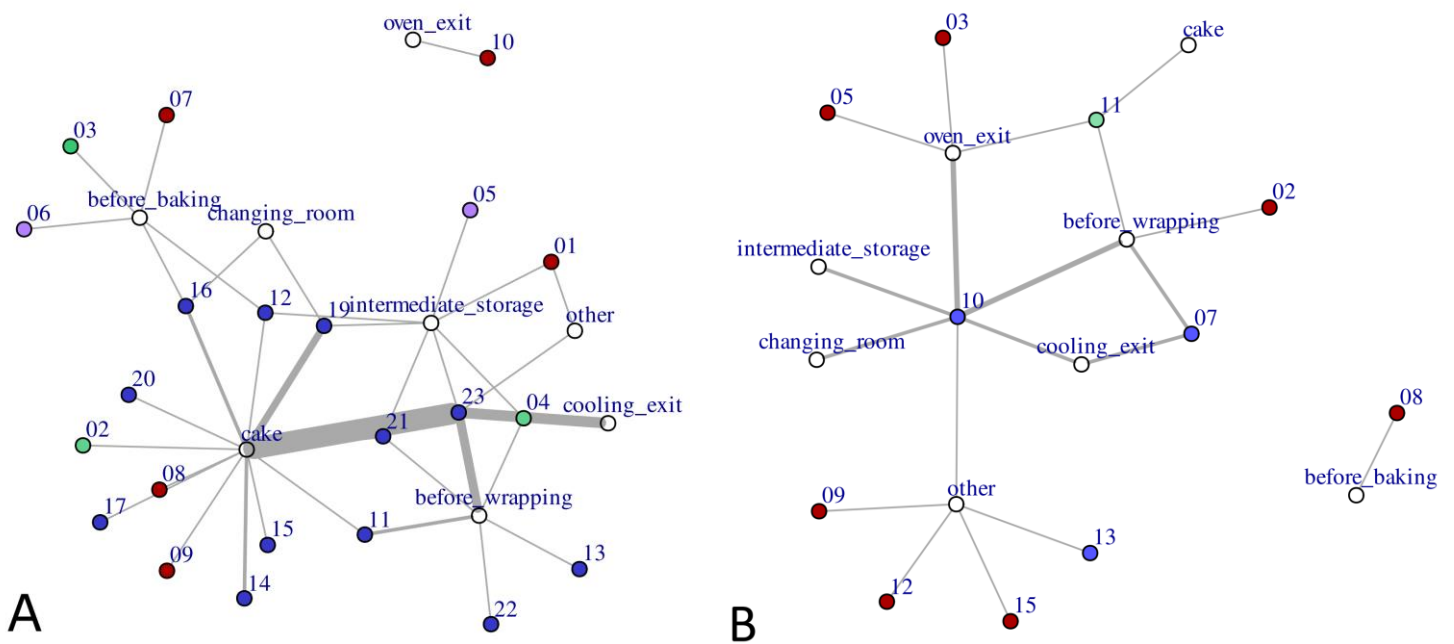


Figure 4. Co-occurrences of *C. sphaerospermum* (A) and *P. brevicompactum* (B) haplotypes within various areas of the bakery. Circular nodes represent haplotypes, while square nodes denote specific bakery areas. The color of each haplotype node indicates its associated population: blue (Population 1), red (Population 2), green (Population 3) and, only for *C. sphaerospermum*, lavender (Population 4). Bakery area nodes are depicted in dark grey. Edge thickness reflects the frequency of co-occurrence between haplotypes and bakery areas, with thicker edges signifying higher co-occurrence frequencies. Haplotype labels are offset to enhance readability, and the proximity of haplotype nodes to specific bakery area nodes suggests shared occurrences in those areas.

Twenty-eight isolates of *P. brevicompactum* were analyzed using 13 SSR markers. The number of alleles per locus ranged from 5 to 10 (Table 4), with an average of 6.85 alleles per locus (Table 5). The average expected heterozygosity (Hexp) across loci was 0.59, and the Simpson's diversity index (1-D) was 0.56, indicating moderate genetic diversity within the overall population. A total of 11 distinct multilocus genotypes (MLGs) were identified among the 28 isolates (Supplementary Table 3).

Table 4. Summary of genetic diversity metrics for each SSR locus analyzed in the *Penicillium brevicompactum* isolate. The table includes the SSR marker name (asterisks indicate SSR markers retained after excluding those with significant linkage disequilibrium, $r_{barD} \geq 0.8$), Simpson's Diversity Index (1-D), Nei's Expected Heterozygosity (Hexp), and Evenness.

Locus	Repeat	Allele	1-D	Hexp	Evenness
PB_SS09*	(TGC) ₃₋₁₇	10.00	0.73	0.76	0.59
PB_SSR13*	(TTC) ₂₋₁₅	7.00	0.56	0.58	0.51
PB_SSR19	(TC) ₁₋₁₂	5.00	0.51	0.53	0.57
PB_SSR21	(AGA) ₁₋₁₆	9.00	0.71	0.73	0.57
PB_SSR22	(CT) ₁₋₁₀	5.00	0.44	0.46	0.52
PB_SSR29	(CA) ₄₋₁₄	6.00	0.51	0.53	0.53
PB_SSR31	(CT) ₄₋₁₉	5.00	0.44	0.46	0.52
PB_SSR32	(GA) ₃₋₂₁	6.00	0.54	0.56	0.58
PB_SSR35	(TC) ₆₋₂₄	6.00	0.69	0.71	0.69
PB_SSR37	(TG) ₀₋₆ (AG) ₅₋₁₅	9.00	0.57	0.59	0.45
PB_SSR43	(AG) ₂₋₁₄	6.00	0.45	0.46	0.49
PB_SSR54	(CT) ₆₋₁₃	6.00	0.48	0.50	0.50
PB_SSR57	(TG) ₅₋₁₅	9.00	0.71	0.73	0.57
mean	-	6.85	0.56	0.59	0.55

Table 5. Summary of genetic diversity and linkage disequilibrium metrics across different *Penicillium brevicompactum* populations. The table includes the population identifier (Population), the number of isolates for each population or overall (Isolate Number), the number of distinct multilocus genotypes identified (MLG), the expected number of MLGs accounting for sampling effort (eMLG), the standard error of the eMLG estimate (SE), Shannon-Wiener diversity index (H), Nei's expected heterozygosity (Hexp), index of association (Ia), and standardized index of association (rbarD).

Genetic cluster	Isolate	MLG	eMLG	SE	H	G	lambda	E.5	Hexp	Ia	rbarD
	number										
1	18	3	2.53	0.512	0.73	1.74	0.426	0.690	0.119	2.75	0.918
2	7	7	7.00	0.000	1.95	7.00	0.857	1.000	0.932	2.96	0.309
3	3	1	1.00	0.000	0.00	1.00	0.000	NaN	0.000	NaN	NaN
Total	28	11	5.46	1.157	1.83	3.88	0.742	0.553	0.585	9.49	0.794

We investigated the population structure of the sampled isolates using the STRUCTURE program. STRUCTURE identified well-defined genetic clusters at K values up to three, with some limited admixture observed between clusters 1 and 2, and between clusters 2 and 3 (Figure 5.B). This suggests the presence of three genetically differentiated clusters. While these clusters may correspond to distinct *P. brevicompactum* populations, the localized and limited nature of our sampling restricts broader conclusions about the species' population structure (Supplementary Figure 4). The deltaK value confirmed that this three-cluster model was the most robust structure in the dataset (Figure 5.C). PCoA supported this structure, although the separation between clusters 1 and 2 was less pronounced, particularly with isolate PB-2169, which is situated between the two clusters. This isolate also displayed significant admixture, as observed in the STRUCTURE analysis (Figure 5.B). The observed diversity levels suggest varying degrees of genetic differentiation across the clusters. Cluster 1, the largest with 18 isolates, showed relatively low genetic diversity, with only 3 multilocus genotypes (MLGs) identified. Cluster 2, comprising 7 isolates, exhibited higher diversity with 7 MLGs, while Cluster 3, which consisted of only 3 isolates, had 1 MLG, but the sample size was too small to draw meaningful conclusions.

Based on the F_{ST} calculations, whether using the full set of 13 SSR markers or the reduced dataset of 23 SSR markers (the reduced dataset was used because F_{ST} calculations are highly sensitive to linkage disequilibrium), genetic cluster 3 remains relatively similar to genetic cluster 2 (Table 6). This similarity suggests a closer relationship between these two genetic clusters, which are, however, still relatively well differentiated from cluster 1. However, it is important to note that the set of markers used is likely too limited to produce fully interpretable results, especially with only two markers.

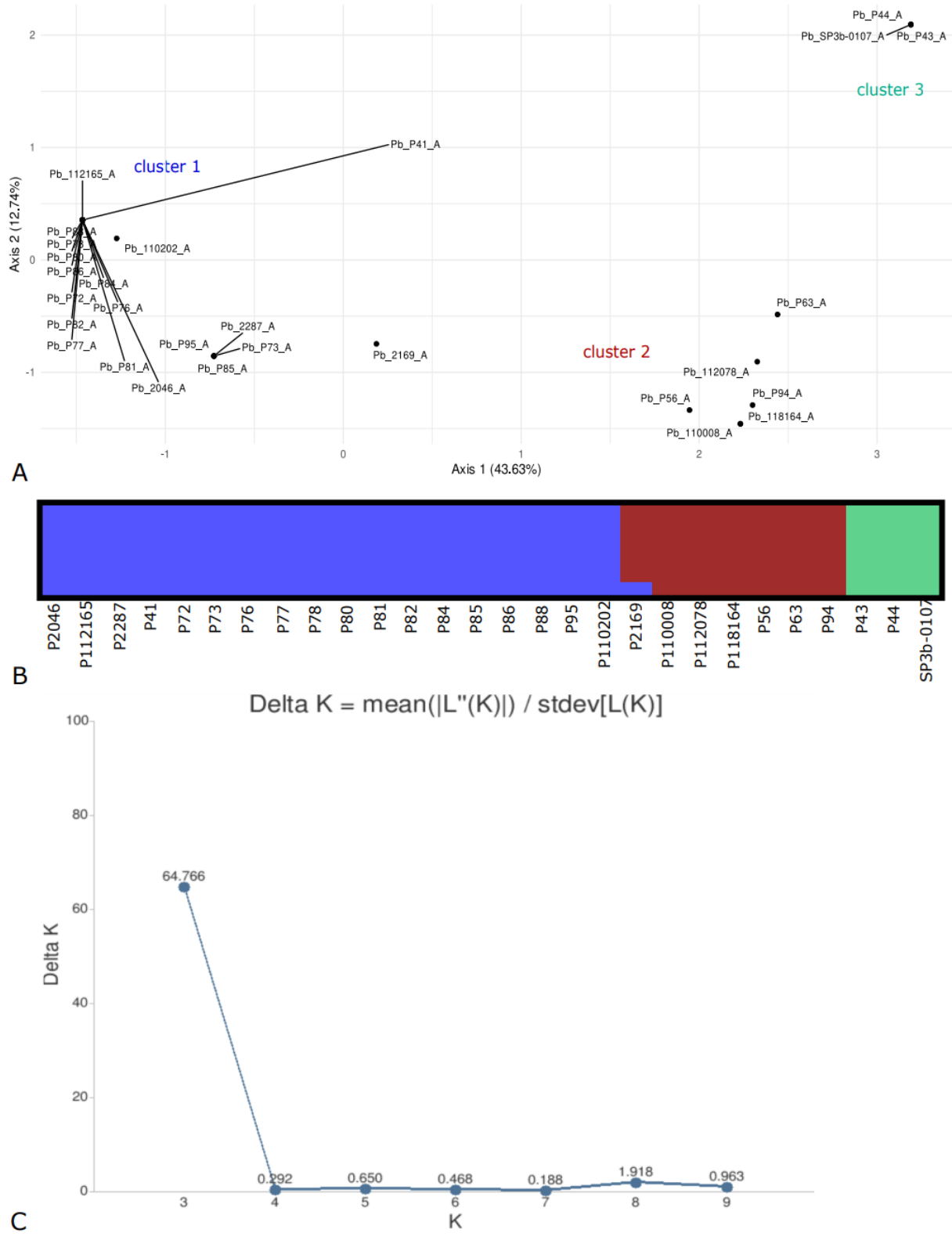


Figure 5. Principal Correspondence Analysis performed using R on the 30 *P. brevicompactum* isolates (A). Detection of the uppermost level of structure of the *P. brevicompactum* dataset. Delta K data obtained using Evanno method and inferred by STRUCTURE (B). Population structure of *P. brevicompactum* (inferred by STRUCTURE for K = 3) (C).

Table 6. Pairwise F_{ST} values representing the genetic differentiation between the three populations in *Penicillium brevicompactum*, identified using 13 SSR markers. Values in parentheses represent F_{ST} values calculated using a reduced dataset of 3 SSR markers, where markers showing significant linkage disequilibrium ($r_{LD} \geq 0.8$) were excluded. F_{ST} values range from 0 to 1, with higher values indicating greater genetic differentiation. "-" denotes comparisons within the same population, where F_{ST} is not applicable.

	Genetic cluster 1	Genetic cluster 2	Genetic cluster 3
Genetic cluster 1	-	0.18 (0.16)	0.32 (0.28)
Genetic cluster 2	0.18 (0.16)	-	0.11 (0.12)
Genetic cluster 3	0.32 (0.28)	0.11 (0.12)	-

To explore the spatial distribution and co-occurrence of *P. brevicompactum* haplotypes within the bakery environment, we performed a co-occurrence network analysis (Figure 4). The analysis revealed a different pattern compared to previous observations with *C. sphaerospermum*. In this case, haplotypes from different genetic clusters also co-occur within specific bakery areas, but the distribution and connections are more diffuse, with no single area serving as a dominant central node.

Areas such as the intermediate storage and cooling exit show multiple connections to various haplotypes, suggesting these locations may play significant roles in the dissemination of *P. brevicompactum* within the bakery. The oven exit and before wrapping areas also exhibit notable connections with the cake node, indicating their potential importance in the spread of contaminants, although the connections are less dense compared to the *C. sphaerospermum* network. Interestingly, the before_baking area shows fewer and weaker connections to the other nodes, suggesting that this zone might contribute less to the overall contamination.

3.3.2. MALDI-TOF MS analysis

The distances between all spectra according to their genetic populations, and the impact of calibration on the separation of these populations, are visualized in Figure 6, which shows the results of the t-SNE analysis before (Figure 6.A.2 and B.2) and after calibration (Figure 6.A.1 and B.1). In general, we observed a good correspondence between spectra from the same isolate and the t-SNE based on MALDI-TOF MS data revealed distinct clusters for both *C. sphaerospermum* and *P. brevicompactum*. The separation observed in the t-SNE analysis was in agreement with the genetic clusters identified through SSR analysis. Furthermore, for *C. sphaerospermum*, t-SNE analysis suggests that MS spectra of isolates belonging to genetic Cluster 1 based on SSRs can be further subdivided into three distinct subclusters, suggesting additional complexity at the intrapopulation level. In contrast, for *P. brevicompactum*, genetic clusters 2 and 3 are less distinguishable in the t-SNE analysis of MS spectra, suggesting that the separation of these clusters is not as clear in this dataset. Overall, the two-step calibration applied in this study improved the separation of spectra, reinforcing the potential of MALDI-TOF MS to differentiate filamentous fungi at the intraspecific level when combined with proper recalibration. This congruence between MALDI-TOF MS and SSR cluster separation highlights the robustness of both techniques for studying fungal population structure.

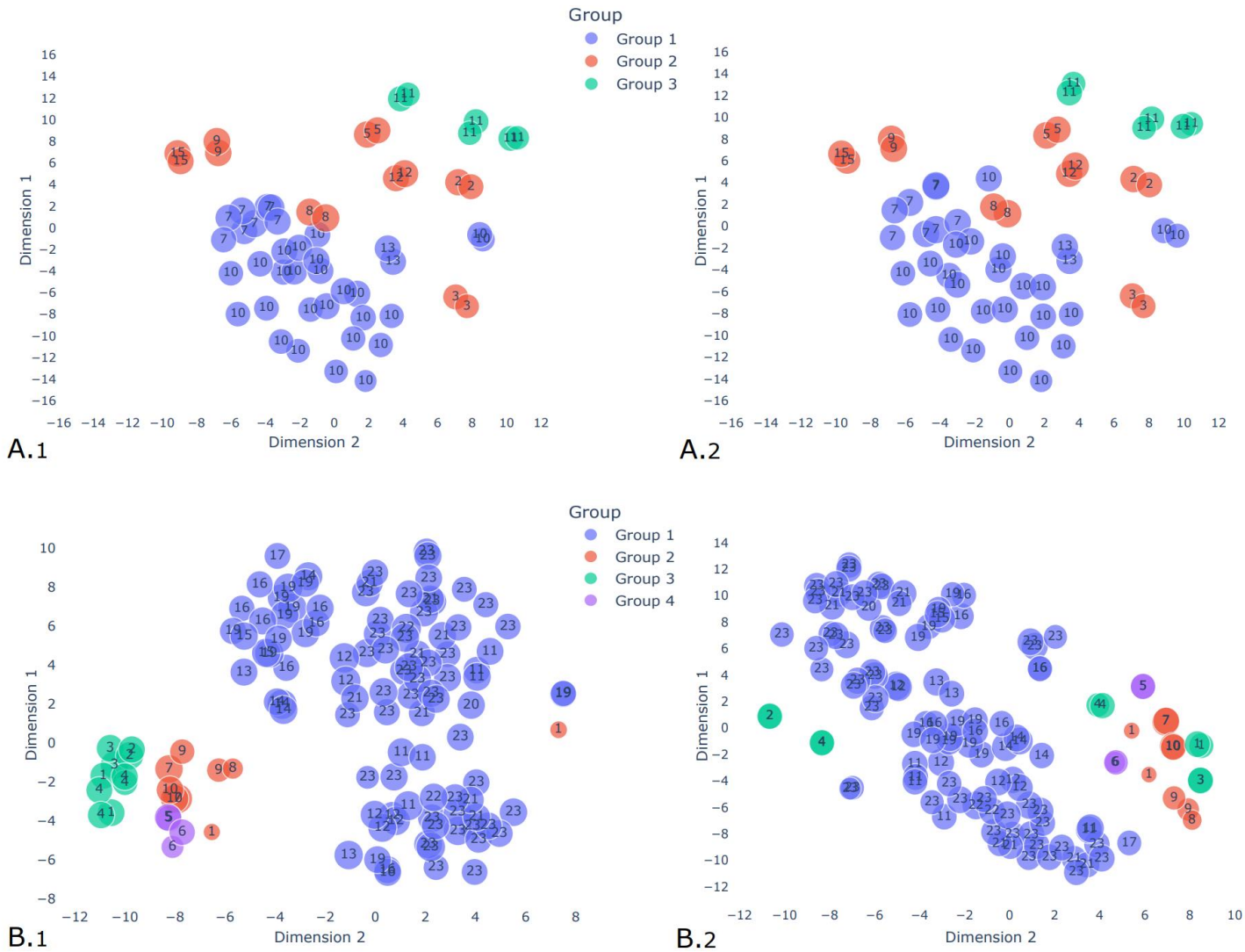


Figure 6. Two-dimensional t-SNE map displaying MALDI-TOF MS spectra from *P. brevicompactum* (A.1 recalibrated dataset, A.2 not-recalibrated dataset) and *C. sphaerospermum* (B.1 recalibrated dataset, B.2 not-recalibrated dataset) isolates. Spectra are colored according to the respective population identified using results obtained via STRUCTURE.

4. Discussion

In this study, we conducted a comprehensive analysis of fungal contamination in a French bakery/pastry factory, investigating both the factory environment and spoiled products. Using a combination of MALDI-TOF MS and SSR-seq, we aimed to trace the sources of contamination and understand the genetic diversity of the prominent spoilage species.

Fungal counts revealed widespread contamination throughout the bakery. Fungal loads were highest in non-pressurized areas, such as the « before baking » and « intermediate storage » areas, where fungal spores likely accumulate due to insufficient airflow control. In contrast, zones like the oven exit and cooling areas, equipped with air filtration systems, had lower contamination levels. This suggests that air control measures effectively reduce mold proliferation, but contamination hotspots remain in areas with less stringent control. In spoiled cakes, contamination was also significant, particularly in products not sprayed with ethanol, while ethanol-treated cakes showed much lower fungal counts. This highlights the importance of surface treatments like ethanol spraying in reducing spoilage risks during storage (Neetoo *et al.*, 2012).

Notably, there were important intra-week and intra-day variations in mold counts, especially in air-controlled areas, and significant but weak correlations were observed between these variables and the frequency of spoiled products at the end of their shelf life. Therefore, an extensive study on the specific events leading to increased mold counts in these areas could be useful to better understand these changes and implement corrective actions. Additionally, work clothes were identified as a potential source of spoilage molds, so better control of personnel movement in these areas would be beneficial. To minimize contamination risks in the production environment, special attention should be paid to preventing the transfer of contaminants between different zones. This can be achieved by implementing traffic flow charts and ensuring that external materials are not introduced into production areas without proper decontamination. Furthermore, improving filtration systems and isolating critical stages of production, such as post-baking, could help mitigate contamination risks. Raw materials entering the factory must also be carefully monitored, as fungal spoilage of products can be linked to contamination of cereals in the field by spores that remain viable in flour (Bernardi et

al., 2019). These fungal spores can later spread through the processing environment, leading to contamination. Thus, controlling both raw material quality and environmental factors is essential for maintaining product safety. The data provided in this study can help identify key control points and refine strategies to better control product spoilage. The use of MALDI-TOF MS combined with sequencing for identifying isolates from this extensive sampling campaign proved to be a quick, reliable, and easily applicable approach in an industrial context.

In terms of species diversity, *Cladosporium* and *Penicillium* species were the dominant molds isolated from both the factory environment and cakes. Specifically, *C. sphaerospermum* and *P. brevicompactum* were frequently identified in all areas of the bakery, confirming their role as key spoilage agents. Other species, such as *Aspergillus* spp., were also found in both the factory environment and spoiled products, while *Wallemia* spp. were only found in spoiled products. These xerotolerant/moderately xerophilic and xerophilic fungi are common in the environment (e.g., air, dust) and frequently involved in bakery product spoilage (Biro et al., 2009 ; Borges Teixeira et al., 2024 ; Chou et al., 2023 ; Le Lay et al., 2016 ; Pitt and Hocking, 2009). Their occurrence can be linked to their prevalence in outdoor and indoor air, as well as in wheat flour, which can serve as a contamination reservoir within the manufacturing environment (Vytrasová et al., 2002). For instance, *Cladosporium* spp. are well adapted to aerial dispersal and are among the most abundant fungi in outdoor and indoor air (Bensch et al., 2018).

It is noteworthy that *A. melanogenum* and *Wallemia* spp. were absent from environmental samples, while *Aspergillus* section *Aspergillus* was rarely identified. Concerning these two taxa, it is not surprising, given their poor growth on the culture media used for environmental sampling (M2Lev and SDCN agar) and the presence of faster-growing fungal species. The application of DNA-based approaches, such as quantitative PCR or ITS metagenetics, could have helped overcome the limitations of our culture-based sampling methods. Regarding *A. melanogenum*, which was sporadically isolated, we observed that it was generally localized on the underside of spoiled products in areas with excess humidity.

Our results showed that fungal species composition varied across different areas of the factory. For example, *Aspergillus* species were mostly confined to the before baking zone, while *Wallemia* spp., known for their xerophilic properties, were primarily found in spoiled products

and not in the factory environment. The NMDS analysis illustrated significant compositional differences between areas such as the pre-baking zone and intermediate storage, which were distinct from other production areas like the cooling exit and packaging areas. These differences likely reflect the influence of various environmental factors such as airflow control, humidity, and human activity.

Focusing on the two most prominent species, *C. sphaerospermum* and *P. brevicompactum*, SSR-seq analysis provided insights into their genetic diversity and potential contamination sources. For *C. sphaerospermum*, 22 distinct multilocus genotypes (MLGs) were identified, with moderate genetic diversity across different clusters. Some clusters, particularly cluster 1, exhibited lower diversity, suggesting the persistence of dominant genotypes within the factory. This raises the possibility of repeated contamination from a common source, potentially from areas with higher fungal counts like the intermediate storage or before_baking zone. Similarly, *P. brevicompactum* showed 11 distinct MLGs across three clusters. While genetic diversity was slightly lower compared to *C. sphaerospermum*, contamination patterns for *P. brevicompactum* were more diffuse, with critical zones like the intermediate storage and cooling exit contributing to the spread of this species. However, the limited number of isolates for *P. brevicompactum* suggests that a larger dataset may be needed to draw more definitive conclusions about its contamination pathways.

The combination of fungal counts, species identification, and genetic diversity analysis revealed important insights into contamination flows within the bakery. The distinct fungal compositions in certain factory areas, particularly the pre-baking and intermediate storage zones, suggest that these areas serve as entry points for fungal spores, either from raw materials or external sources such as packaging. The genetic analysis supports these findings, showing that the cooling exit and packaging area before wrapping are critical points of contamination for *C. sphaerospermum*, while the intermediate storage plays a similar role for *P. brevicompactum*. Despite air filtration and pressurization systems, these post-baking zones remain vulnerable to mold contamination, indicating the need for more stringent control measures in these areas. In addition to improving airflow control, our results highlight the importance of monitoring raw materials for fungal contamination, as cereal-based ingredients can act as reservoirs for mold spores. Regular screening of incoming materials and stricter decontamination protocols for packaging are essential for reducing contamination risks.

Furthermore, while ethanol spraying proved effective in reducing fungal spoilage on cakes, additional preservation strategies, such as the use of natural biopreservatives, may be needed to combat resistant species like *Wallemia* spp.

In the last part of this study, we evaluated MALDI-TOF MS as a rapid tool for the source-tracking of *C. sphaerospermum* and *P. brevicompactum*. Using an unsupervised approach based on t-SNE analysis of recalibrated MS spectra, we showed that there was a good agreement between SSR results at the population level as previously observed for *P. roqueforti* (Quero et al., 2020). While this is a promising result, it would be of interest to assess the discriminative power of MALDI-TOF MS using a supervised approach, i.e., after database construction and evaluation of classification performances using cross-validation and external validation. Furthermore, studying the performance of MALDI-TOF MS at the haplotype level would be necessary for accurate source-tracking of spoilage molds.

Supplementary materials

Supplementary Figure 1. Simplified factory's map

Supplementary Figure 2. Correlogram showing significant Spearman's correlations ($p < 0.05$) between airborne fungal counts and frequency of spoiled products with or without ethanol spraying. Pen, *Penicillium* spp.; Clado, *Cladosporium* spp.

Supplementary Table 1. Diversity of spoilage molds isolated from spoiled cakes.

Supplementary Table 2. Diversity of spoilage molds isolated from various areas in the bakery and pastry factory.

Supplementary Table 3. List of fungal isolates, specifying the species (*Cladosporium sphaerospermum* or *Penicillium brevicompactum*), the sample origin, and the associated haplotype and genetic cluster. Both the haplotype and genetic cluster are species-specific.

Supplementary Figure 3. Population structure of *Cladosporium sphaerospermum*. Barplots corresponding to K values from 2 to 10.

Supplementary Figure 4. Population structure of *Penicillium brevicompactum*. Barplots corresponding to K values from 2 to 10.

Conflicts of interest

N.R., V.G. and G.P. are employees of bioMérieux, a company developing and selling in vitro diagnostic assays including the VITEK MS used in this study. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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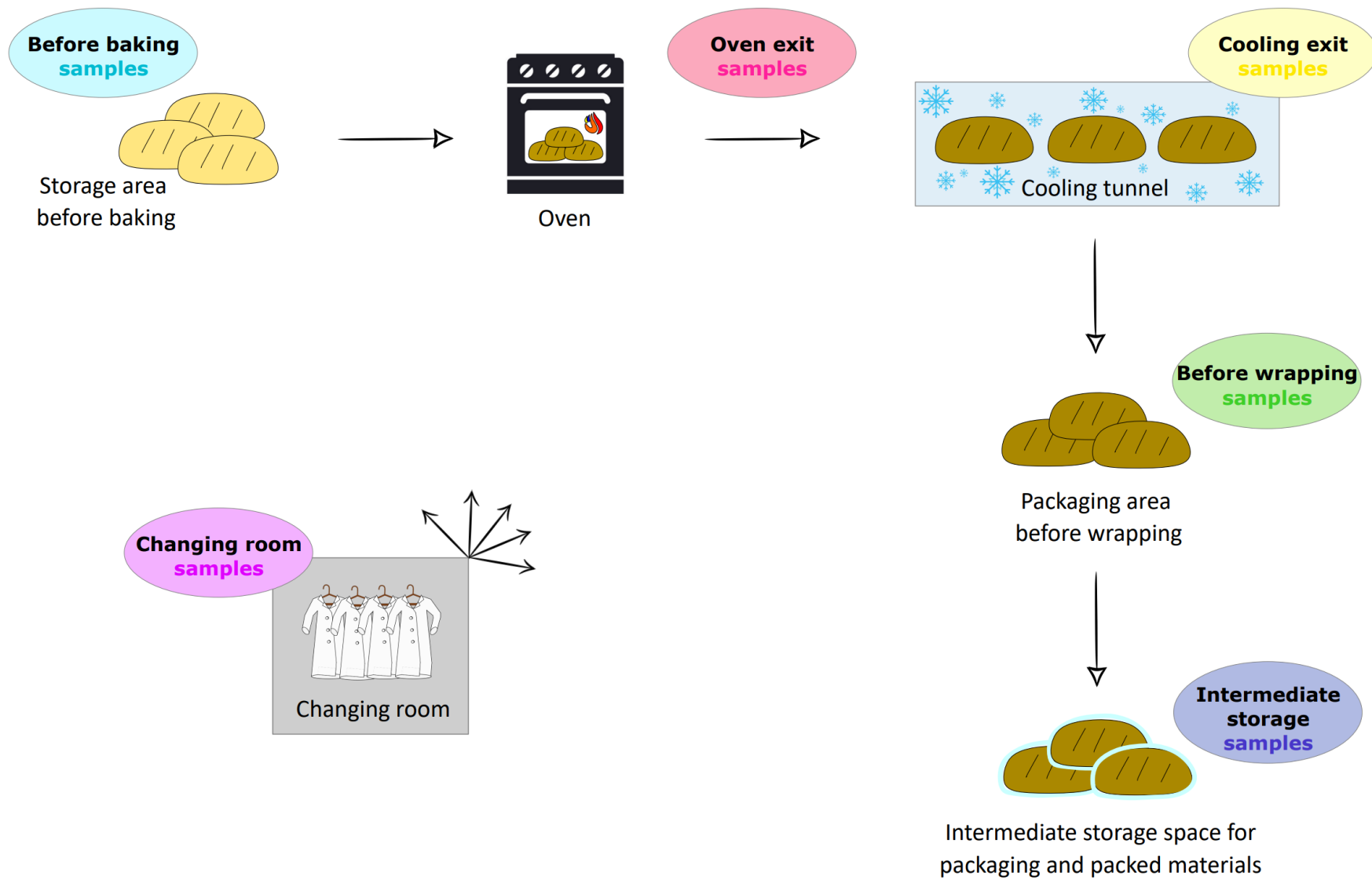
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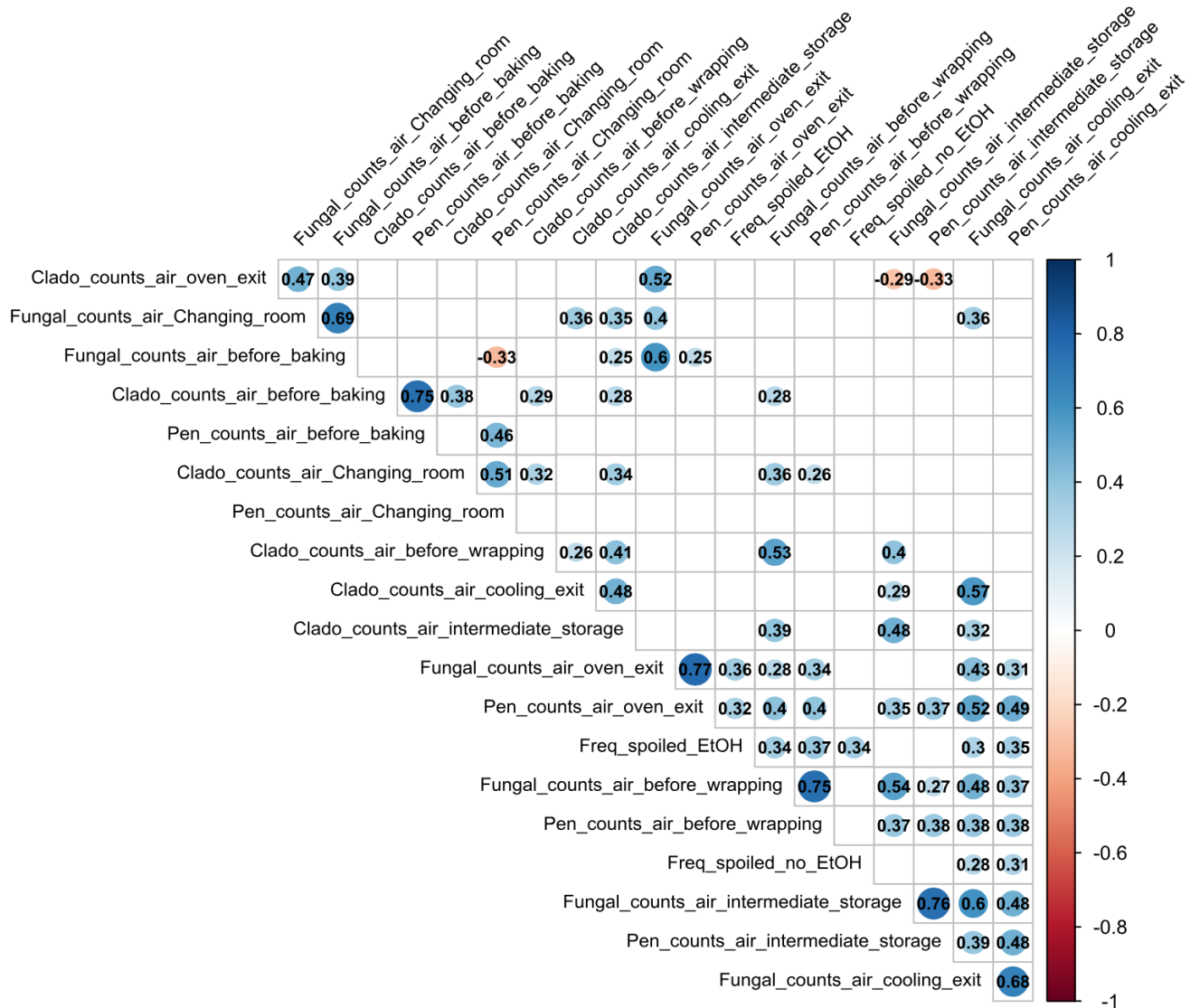
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Supplementary Figure 1. Simplified factory's map



Supplementary Figure 2. Correlogram showing significant Spearman's correlations ($p < 0.05$) between airborne fungal counts and frequency of spoiled products with or without ethanol spraying. Pen, *Penicillium* spp.; Clado, *Cladosporium* spp.

Supplementary Table 1. Diversity of spoilage molds isolated from spoiled cakes.

Taxon	Isolation source (number of isolates)		Total number
	Cake with ethanol spraying	Cake without ethanol spraying	
<i>Aspergillus cibarius</i>	1		1
<i>Aspergillus destruens</i>		1	1
<i>Aspergillus mallochii</i>		1	1
<i>Aspergillus montevicensis</i>		1	1
<i>Aspergillus niveoglaucus</i>		1	1
<i>Aspergillus proliferans</i>	1		1
<i>Aspergillus</i> section <i>Aspergillus</i>	1	5	6
<i>Aspergillus</i> section <i>Flavi</i>		3	3
<i>Aureobasidium melanogenum</i>	2	3	5
<i>Cladosporium domesticum</i>		1	1
<i>Cladosporium halotolerans</i>		1	1
<i>Cladosporium inversicolor</i>		1	1
<i>Cladosporium psychrotolerans</i>		1	1
<i>Cladosporium pulvericola</i>		1	1
<i>Cladosporium sphaerospermum</i>	8	55	63
<i>Paraphaeosphaeria verruculosa/parmeliae</i>		1	1
<i>Penicillium bialowiezense</i>	1	2	3
<i>Penicillium brevicompactum</i>		11	11
<i>Penicillium buchwaldii</i>	1		1
<i>Penicillium chrysogenum</i>	1	4	5
<i>Penicillium citreonigrum</i>	1		1
<i>Penicillium citrinum</i>		2	2
<i>Penicillium corylophilum</i>	1	2	3

Taxon	Isolation source (number of isolates)		Total number
	Cake with ethanol spraying	Cake without ethanol spraying	
<i>Penicillium crustosum</i>		4	4
<i>Penicillium palitans</i>		1	1
<i>Penicillium paneum</i>		1	1
<i>Penicillium salamii</i>		1	1
<i>Penicillium solitum</i>		1	1
<i>Penicillium</i> sp.	1		1
<i>Penicillium spathulatum</i>		1	1
<i>Wallemia sebi</i> species complex	6	22	28
Total number	25	128	153

Supplementary Table 2. Diversity of spoilage molds isolated from various areas in the bakery and pastry factory.

Taxon	Isolation source (number of isolates)							Total
	Changing room	before baking	oven exit	cooling exit	before wrapping	intermediate storage	working clothes	
<i>Aspergillus brasiliensis</i>	0	0	0	0	0	0	1	1
<i>Aspergillus repens</i>	0	0	0	0	1	2	0	3
<i>Aspergillus section Circumdati</i>	0	0	0	0	0	0	1	1
<i>Aspergillus section Flavi</i>	0	1	0	0	0	0	0	1
<i>Cladosporium allicinum</i>	0	0	2	1	0	2	2	7
<i>Cladosporium brunhei</i>	0	0	4	3	2	4	4	17
<i>Cladosporium cladosporioides complex</i>	3	3	6	5	3	4	8	32
<i>Cladosporium halotolerans</i>	0	0	0	1	1	0	1	3
<i>Cladosporium herbarum</i>	0	0	0	0	0	0	1	1
<i>Cladosporium langeronii</i>	0	0	2	0	0	0	0	2
<i>Cladosporium psychrotolerans</i>	1	0	1	1	1	3	1	8
<i>Cladosporium ramotenellum</i>	1	1	2	1	2	1	3	11
<i>Cladosporium sphaerospermum</i>	6	6	4	8	9	4	6	43

Taxon	Isolation source (number of isolates)							Total
	Changing room	before baking	oven exit	cooling exit	before wrapping	intermediate storage	working clothes	
<i>Cladosporium vicinum</i>	0	0	1	0	0	0	0	1
<i>Eurotium amstelodami</i>	0	1	0	0	0	0	0	1
<i>Exophiala lecanii-corni</i>	0	0	0	0	0	1	0	1
<i>Fusarium cerealis</i>	0	1	0	0	0	0	0	1
<i>Humicola brunnea</i> var. <i>brunnea</i>	0	0	0	0	0	0	1	1
<i>Ochroconis humicola</i>	0	0	1	0	0	0	0	1
<i>Paecilomyces variotii</i> complex	0	0	0	0	2	0	0	2
<i>Penicillium aurantiogriseum</i>	0	0	0	1	0	0	0	1
<i>Penicillium bialowiezense</i>	1	1	3	2	2	1	1	11
<i>Penicillium bifforme</i>	1	0	0	1	1	0	0	3
<i>Penicillium brevicompactum</i>	4	0	8	9	8	1	3	33
<i>Penicillium buchwaldii</i>	0	0	0	0	0	0	1	1
<i>Penicillium camemberti</i> complex	0	2	0	0	0	2	0	4
<i>Penicillium citrinum</i>	0	0	0	0	0	0	1	1

Taxon	Isolation source (number of isolates)							Total
	Changing room	before baking	oven exit	cooling exit	before wrapping	intermediate storage	working clothes	
<i>Penicillium corylophilum</i>	0	0	0	0	0	2	0	2
<i>Penicillium crustosum</i>	0	2	4	3	5	1	7	22
<i>Penicillium echinulatum</i>	0	0	0	1	0	0	0	1
<i>Penicillium expansum</i>	0	0	0	0	0	1	0	1
<i>Penicillium glabrum</i>	0	0	0	0	0	0	1	1
<i>Penicillium janczewskii</i>	0	0	0	1	0	0	1	2
<i>Penicillium nordicum</i>	0	0	0	0	0	1	0	1
<i>Penicillium paneum</i>	0	0	0	1	0	0	0	1
<i>Penicillium polonicum</i>	0	0	0	1	0	0	0	1
<i>Penicillium rubens</i>	0	1	0	1	2	2	1	7
<i>Penicillium rugulosum</i>	0	0	0	0	0	0	1	1
<i>Penicillium solitum</i>	0	1	0	1	0	0	0	2
<i>Rhizopus microsporus complex</i>	0	1	0	0	0	0	0	1
Total	17	21	38	42	39	32	46	235

Supplementary Table 3. List of fungal isolates, specifying the species (*Cladosporium sphaerospermum* or *Penicillium brevicompactum*), the sample origin, and the associated haplotype and genetic cluster. Both the haplotype and genetic cluster are species-specific.

Isolate	Species	Sample origin	Haplotype	Genetic cluster
1949	<i>C. sphaerospermum</i>	cake	19	1
1950	<i>C. sphaerospermum</i>	cake	19	1
2123	<i>C. sphaerospermum</i>	cake	8	2
2126	<i>C. sphaerospermum</i>	cake	9	2
2149	<i>C. sphaerospermum</i>	cake	17	1
2152	<i>C. sphaerospermum</i>	cake	14	1
2154	<i>C. sphaerospermum</i>	cake	12	1
2155	<i>C. sphaerospermum</i>	cake	14	1
2156	<i>C. sphaerospermum</i>	cake	11	1
2167	<i>C. sphaerospermum</i>	cake	23	1
2283	<i>C. sphaerospermum</i>	changing_room	19	1
2286	<i>C. sphaerospermum</i>	before_baking	6	4
2296	<i>C. sphaerospermum</i>	cake	23	1
2298	<i>C. sphaerospermum</i>	cake	23	1
2300	<i>C. sphaerospermum</i>	cake	16	1
2301	<i>C. sphaerospermum</i>	cake	23	1
113079	<i>C. sphaerospermum</i>	other	1	2
113080	<i>C. sphaerospermum</i>	other	23	1
C10	<i>C. sphaerospermum</i>	before_baking	16	1
C130	<i>C. sphaerospermum</i>	before_wrapping	11	1
C132	<i>C. sphaerospermum</i>	intermediate_storage	19	1
C133	<i>C. sphaerospermum</i>	cooling_exit	23	1

Isolate	Species	Sample origin	Haplotype	Genetic cluster
C147	<i>C. sphaerospermum</i>	cooling_exit	23	1
C162	<i>C. sphaerospermum</i>	cooling_exit	23	1
C166	<i>C. sphaerospermum</i>	oven_exit	10	2
C167	<i>C. sphaerospermum</i>	before_wrapping	22	1
C168	<i>C. sphaerospermum</i>	before_wrapping	23	1
C169	<i>C. sphaerospermum</i>	intermediate_storage	4	3
C171	<i>C. sphaerospermum</i>	before_wrapping	4	3
C18	<i>C. sphaerospermum</i>	intermediate_storage	12	1
C19	<i>C. sphaerospermum</i>	intermediate_storage	1	3
C205	<i>C. sphaerospermum</i>	before_wrapping	23	1
C212	<i>C. sphaerospermum</i>	cooling_exit	23	1
C222	<i>C. sphaerospermum</i>	before_wrapping	13	1
C231	<i>C. sphaerospermum</i>	before_baking	12	1
C249	<i>C. sphaerospermum</i>	intermediate_storage	5	4
C40	<i>C. sphaerospermum</i>	before_wrapping	23	1
C41	<i>C. sphaerospermum</i>	before_wrapping	21	1
C48	<i>C. sphaerospermum</i>	before_wrapping	11	1
C51	<i>C. sphaerospermum</i>	before_baking	3	3
C7	<i>C. sphaerospermum</i>	changing_room	16	1
C73	<i>C. sphaerospermum</i>	cooling_exit	23	1
C78	<i>C. sphaerospermum</i>	cooling_exit	23	1
C80	<i>C. sphaerospermum</i>	before_wrapping	23	1
C81	<i>C. sphaerospermum</i>	intermediate_storage	21	1
C88	<i>C. sphaerospermum</i>	intermediate_storage	23	1

Isolate	Species	Sample origin	Haplotype	Genetic cluster
C9	<i>C. sphaerospermum</i>	before_baking	7	2
C95	<i>C. sphaerospermum</i>	before_wrapping	23	1
P1-2307	<i>C. sphaerospermum</i>	cake	19	1
P2-2307	<i>C. sphaerospermum</i>	cake	23	1
SP1-0107	<i>C. sphaerospermum</i>	cake	15	1
SP1-0807	<i>C. sphaerospermum</i>	cake	23	1
SP1-2806	<i>C. sphaerospermum</i>	cake	19	1
SP2-0508am	<i>C. sphaerospermum</i>	cake	23	1
SP2-0807	<i>C. sphaerospermum</i>	cake	23	1
SP2-2307	<i>C. sphaerospermum</i>	cake	16	1
SP3-0807	<i>C. sphaerospermum</i>	cake	23	1
SP4-0807	<i>C. sphaerospermum</i>	cake	21	1
SP4-2806	<i>C. sphaerospermum</i>	cake	21	1
SP5-2307	<i>C. sphaerospermum</i>	cake	23	1
SP6-0508m	<i>C. sphaerospermum</i>	cake	20	1
SP6-2307	<i>C. sphaerospermum</i>	cake	2	3
SP8-0508am	<i>C. sphaerospermum</i>	cake	23	1
SP9b-0508am	<i>C. sphaerospermum</i>	cake	23	1
P41	<i>P. brevicompactum</i>	before_wrapping	10	1
P43	<i>P. brevicompactum</i>	oven_exit	11	3
P44	<i>P. brevicompactum</i>	before_wrapping	11	3
P56	<i>P. brevicompactum</i>	oven_exit	3	2
P63	<i>P. brevicompactum</i>	oven_exit	5	2
P72	<i>P. brevicompactum</i>	oven_exit	10	1

Isolate	Species	Sample origin	Haplotype	Genetic cluster
P73	<i>P. brevicompactum</i>	cooling_exit	7	1
P76	<i>P. brevicompactum</i>	changing_room	10	1
P77	<i>P. brevicompactum</i>	oven_exit	10	1
P78	<i>P. brevicompactum</i>	oven_exit	10	1
P80	<i>P. brevicompactum</i>	cooling_exit	10	1
P81	<i>P. brevicompactum</i>	before_wrapping	10	1
P82	<i>P. brevicompactum</i>	before_wrapping	10	1
P84	<i>P. brevicompactum</i>	cooling_exit	10	1
P85	<i>P. brevicompactum</i>	before_wrapping	7	1
P86	<i>P. brevicompactum</i>	intermediate_storage	10	1
P88	<i>P. brevicompactum</i>	intermediate_storage	10	1
P94	<i>P. brevicompactum</i>	before_wrapping	2	2
P95	<i>P. brevicompactum</i>	before_wrapping	7	1
2046	<i>P. brevicompactum</i>	changing_room	10	1
2169	<i>P. brevicompactum</i>	before_baking	8	2
2287	<i>P. brevicompactum</i>	cooling_exit	7	1
SP3b 01.07	<i>P. brevicompactum</i>	cake	11	3
UBOCC-A-110202	<i>P. brevicompactum</i>	other	13	1
UBOCC-A-110008	<i>P. brevicompactum</i>	other	15	2
UBOCC-A-112078	<i>P. brevicompactum</i>	other	12	2
UBOCC-A-112165	<i>P. brevicompactum</i>	other	10	1
UBOCC-A-118164	<i>P. brevicompactum</i>	other	9	2

K=2



K=3



K=4



K=5



K=6



K=7



K=8



K=9



K=10



Supplementary Figure 3. Population structure of *Cladosporium sphaerospermum*. Barplots corresponding to K values from 2 to 10.

K=2



K=3



K=4



K=5



K=6



K=7



K=8



K=9



K=10



Supplementary Figure 4. Population structure of *Penicillium brevicompactum*. Barplots corresponding to K values from 2 to 10.

III. Résultats supplémentaires : Différentiation des isolats de *Cladosporium sphaerospermum* et de *Penicillium brevicompactum* grâce à la spectrométrie de masse MALDI-TOF : analyse supervisée

Afin d'évaluer le pouvoir discriminant de la spectrométrie de masse MALDI-TOF MS au niveau intra-espèce, en complément de l'approche non-supervisée (t-SNE), une approche supervisée a été entreprise. Cette méthode nécessite, comme dans le cas de l'approche non-supervisée, une recalibration. Elle est basée sur la construction d'une base de données spectrales pour chacune des espèces considérées, c'est-à-dire *C. sphaerospermum* et *P. brevicompactum*. Les performances d'attribution au niveau populationnel sont alors estimées par validation croisée, selon la procédure décrite par Rolland et al. (2024). Pour construire la base de données, 48 et 20 isolats appartenant respectivement aux jeux de données de *C. sphaerospermum* et *P. brevicompactum* et appartenant aux différentes populations déterminées par l'analyse SSR-seq ont été utilisés, tandis que 16 isolats de *C. sphaerospermum* et 10 isolats de *P. brevicompactum* non-utilisés pour la construction de la base de données ont été employés pour la validation externe. De plus, 27 et 18 isolats de *C. sphaerospermum* et *P. brevicompactum*, pour lesquels le génotypage par SSR-seq a échoué, ont également été inclus afin d'évaluer plus largement le jeu de données. En raison du délai de réalisation de cette analyse, les résultats ne sont pas inclus dans l'article précédemment exposé mais seront inclus par la suite.

Discussion et perspectives

Comme souligné dans ce manuscrit, les organismes fongiques font partie d'un vaste groupe englobant des espèces dont les impacts peuvent être autant favorables que défavorables pour les activités humaines. En effet, les levures et les champignons filamenteux sont à même de coloniser une large gamme de substrats, e.g. produits pharmaceutiques, cosmétiques, matrices alimentaires diverses. Ils sont ainsi utilisés pour leur capacité à améliorer les propriétés organoleptiques et nutritionnelles, telles que la teneur en protéines, et/ou à prolonger la durée de conservation des denrées grâce au processus de la fermentation, c'est notamment le cas d'espèces ou de populations fongiques domestiquées (Amara and El-Baky, 2023; Gibbons and Rinker, 2015; Nout, 2007; Pitt and Hocking, 2009). Néanmoins, les champignons jouent également un rôle significatif, chaque année, dans les pertes et gaspillages alimentaires à l'échelle mondiale (Cederberg and Sonesson, 2011; Davies et al., 2021; Pitt and Hocking, 2009). Leur aptitude à coloniser une vaste gamme de matières premières et de produits alimentaires transformés tout au long de la chaîne alimentaire engendre des pertes financières substantielles (Akimowicz and Bucka-Kolendo, 2020; Food and Agriculture Organization of the United Nations (FAO), 2023). Hormis l'altération des produits, le pouvoir mycotoxinogène de certaines moisissures présente un risque sanitaire majeur pour l'homme et les animaux d'élevage. Certains organismes sont également à l'origine d'infections opportunistes chez les personnes immunodéprimés (Köhler et al., 2017; Latgé and Calderone, 2002).

La maîtrise des agents producteurs de mycotoxines est désormais essentielle pour garantir la sécurité et la qualité des produits ainsi que des environnements de production. L'identification des moisissures constitue donc une étape cruciale dans le contrôle de la qualité et de la sécurité des denrées alimentaires (Pinheiro et al., 2019; Rokas, 2022). Dans ce contexte, des nouvelles approches de mesure physique, hautement résolutive, ont émergé. La spectrométrie de masse MALDI-TOF s'est imposée ces dernières années grâce à ses nombreux atouts, parmi lesquels sa rapidité, sa fiabilité, son faible coût, ainsi que la possibilité de l'utiliser sans disposer de compétences spécialisées. Cette approche repose sur la mise en évidence de différents biomarqueurs différenciés par leur rapport masse/charge, tels que les peptides, les protéines (protéines ribosomales), les glycoprotéines et les oligonucléotides, voire les lipides. La finalité est l'obtention d'une empreinte spectrale spécifique d'une espèce microbienne pouvant être comparée à une bibliothèque spectrale de référence (Croxatto et al., 2012). Cette méthode a tout d'abord été appliquée aux bactéries avant d'être étendue aux levures et aux champignons filamenteux (Deak et al., 2015; Patel, 2019; Quéro, 2018). Elle est désormais

employée pour l'identification des microorganismes, bactéries et levures, en routine (Akimowicz and Bucka-Kolendo, 2020).

La spectrométrie de masse MALDI-TOF souffre néanmoins de certaines limites dans le cadre des analyses mycologiques (Dupont et al., 2019). En effet, cette approche est tributaire des bases de données spectrales et de leur exhaustivité. Or, le panel d'espèces fongiques incluses dans les bases de données commerciales reste restreint (Girard et al., 2023; Quéro et al., 2018a). Comme évoqué par Chalupová et al. (2014), l'étude des organismes fongiques demeure complexe en raison d'une compréhension insuffisante de l'ensemble des mécanismes de spéciation fongique et de leurs liens avec la biologie des populations, l'écologie, les processus d'évolution et la phylogénie.

Le premier axe de ces travaux de recherche était consacré à l'enrichissement d'une base de données commerciale afin de répondre aux nouveaux enjeux de l'industrie agroalimentaire. L'extension d'une base dédiée à l'identification rapide des organismes fongiques par spectrométrie de masse MALDI-TOF a nécessité le respect de critères stricts, indispensables pour assurer la justesse¹ et la fidélité² des performances d'identification.

Dans un premier temps, comme abordé précédemment, une sélection rigoureuse d'espèces d'intérêt a été établie grâce à une étude de la littérature. Les critères de sélection incluaient notamment la fréquence d'apparition de l'espèce dans les denrées alimentaires, leur capacité à se développer sur divers types de supports alimentaires, le pouvoir mycotoxinogène ainsi que les réclamations éventuelles des utilisateurs de l'outil. Par ailleurs, avant d'entamer les manipulations, la versatilité de la taxonomie a été scrupuleusement prise en compte, tant pour l'ajout de nouvelles espèces que pour la mise à jour de celles déjà présentes dans la base de données.

Ensuite, il est fondamental d'intégrer à la fois de la diversité génétique et technique. D'une part, les souches d'une même espèce peuvent présenter un certain niveau de diversité. Selon Girard et al. (2023), il est recommandé d'ajouter une dizaine de souches afin de représenter de

¹ Justesse : Ecart de l'accord entre la moyenne d'un nombre infini de valeurs mesurées répétées et une valeur de référence (Guide ISO/CEI 99).

² Fidélité : Ecart de l'accord entre les indications ou les valeurs mesurées obtenues par des mesurages répétés du même objet ou d'objets similaires dans des conditions spécifiées (Guide ISO/CEI 99).
(Organisation Internationale de Normalisation (ISO) and Commission Electrotechnique Internationale (CEI), 2007)

façon optimale cette hétérogénéité. Cet optimum n'étant pas toujours réalisable, d'autres mesures visant à garantir la qualité de la base développée ont également été intégrées, telles que l'ajout de souches types, le respect aspects réglementaires en matières d'exploitation de ressources biologiques (e.g. protocole Nagoya sur l'Accès et Partage des Avantages découlant de l'utilisation des ressources génétiques et des connaissances traditionnelles associées (APA), lois spécifiques à certains pays (Ministère de la Transition Ecologique et de la Cohésion des Territoires, 2024)) et une caractérisation rigoureuse, ce qui complexifie l'obtention de souches jugées adéquates. A cet égard, un maximum de 7 souches par espèce a été atteint pour certaines d'entre elles, lors de ce travail de développement. Les difficultés liées à l'acquisition de multiples souches pour certaines espèces, peu recensées, nouvellement décrites ou non-utilisables en raison du cadre réglementaire, ont apporté une complexité nouvelle à l'extension de la base de données. A titre d'exemple, *Bisifusarium tonghuanum*, faisant partie du complexe *Bisifusarium dimerum* a été isolée et décrite en Chine en 2017 et non déposée dans une mycothèque accessible (Sun et al., 2017). Il n'était donc pas possible d'avoir accès à la seule souche recensée à l'heure actuelle. Dans le cadre des présents travaux, certaines espèces appartenant à des complexes n'étaient représentées en base de données que par une seule souche, alors qu'un minimum de deux est requis. Afin de pallier cette limitation, le choix a été fait de regrouper ces souches uniques avec des espèces très proches sur le plan taxonomique. Cette approche visait à assurer une couverture plus complète des complexes d'espèces concernés n'impliquant pas l'exclusion de ces espèces « incomplètes ». C'est notamment le cas des espèces *Bisifusarium nectrioides*, *Mucor bainieri*, *Mucor ramosissimus* et *Mucor variicolumellatus*. Par exemple, des représentations t-SNE ont été réalisées pour l'ensemble des spectres des souches du complexe *Fusarium dimerum*, révélant un mélange des spectres des espèces *B. nectrioides* et *B. delphinooides*. Ainsi, il a été décidé d'inclure ces espèces en base en les associant grâce à un slashline (espèces regroupées), permettant ainsi de pallier l'insuffisance de diversité. Normand et al. (2013) proposent par ailleurs une autre solution dans cette configuration, elle consiste à multiplier les réplicats biologiques. Cette méthode n'a pas été choisie afin de respecter les critères rigoureux établis pour l'extension de la base VITEK® MS V3.4. D'autre part, la diversité technique doit également être prise en compte, à travers un plan d'expérience intégrant divers milieux provenant de différents fournisseurs ainsi que des durées d'incubation variables pour les moisissures.

La base VITEK® MS V3.4 commercialisée par bioMérieux a ainsi été étendue grâce à 380 souches appartenant à 133 espèces, 119 correspondaient à de toutes nouvelles espèces,

tandis que 14 concernaient des espèces déjà incluses, pour lesquelles des souches ont été ajoutées. Parmi les espèces sélectionnées, cinq complexes d'espèces distincts ont également été ciblés pour une adjonction en base : *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Fusarium dimerum*, *Mucor circinelloides* et la section *Nigri* d'*Aspergillus*. Quéro et al. (2018) avait précédemment démontré la faisabilité de l'identification d'espèces au sein de complexes, et ce, à travers son travail sur la section *Flavi* du genre *Aspergillus*. L'objectif était donc de poursuivre dans cette démarche en procédant à l'ajout de nouveaux complexes d'espèces incluant des pathogènes opportunistes, des agents responsables de l'anthracnose et des espèces potentiellement mycotoxinogènes. Ces espèces sont difficilement différenciables à l'aide de méthodes dites « conventionnelles » (Balasundaram et al., 2015). Les performances très prometteuses obtenues ont démontré la capacité du MALDI-TOF MS à différencier des espèces proches d'un point de vue taxonomique et phénotypique. Il est intéressant de noter que l'inclusion exhaustive des espèces de la section *Nigri* d'*Aspergillus* a permis de travailler à nouveau sur *A. lacticoffeatus* et *A. foetidus* qui avaient respectivement des taux de 62,86% et 25,93% d'identification correcte lors de l'étape de validation croisée (Quéro et al., 2018) et plafonnent à présent à 100%. Cette importante amélioration a notamment été soutenue par l'étude récente de Bian et al. (2022). Nous avons donc remanié la taxonomie en nous basant sur cette nouvelle approche de la section *Nigri*. Cela démontre les connaissances limitées dont nous disposons sur le monde fongique, les données biologiques, phylogénétiques, écologiques et taxonomiques. Ces éléments sont en constante évolution soulignant l'importance d'efforts constants quant à l'inclusion de nouvelles espèces en base ainsi que le suivi régulier des changements de classification taxonomique. Il serait également pertinent de poursuivre l'ajout de complexes, par exemple le complexe *Colletotrichum boniense*, la section *Flavi* d'*Aspergillus* ou encore le complexe *Wallemia sebi* pourraient être à compléter dans les versions ultérieures de la base de données spectrales. Enfin, d'autres secteurs font mention de besoin en termes d'inclusion d'espèces microbiennes environnementales et plus particulièrement de champignons contaminant les eaux potables (Grandhay et al., 2024).

Le second axe s'est porté sur l'application de la spectrométrie de masse MALDI-TOF à l'identification et le typage des contaminants fongiques en milieu industriel. La spectrométrie de masse MALDI-TOF a déjà été utilisée avec succès pour le typage et l'identification au niveau intra-spécifique de certaines bactéries, levures et moisissures et ce également dans des contextes industriels (Lauterbach et al., 2017; Quéro et al., 2020). Nous avons donc souhaité déterminer le pouvoir discriminant de cette méthode dans le cas d'autres moisissures d'intérêt alimentaire.

Une première preuve de contexte avait par ailleurs été réalisée par Quéro et al. (2020) grâce à la distinction au MALDI-TOF MS de populations au sein d'une collection d'isolats de *Penicillium roqueforti*. L'analyse approfondie de la contamination fongique a été réalisée dans une usine de BVP, en examinant à la fois l'environnement de l'usine et les produits altérés. Tout d'abord, les espèces identifiées dans notre étude figurent parmi les principaux agents responsables de l'altération des produits de boulangerie (Le Lay et al., 2016; Pitt and Hocking, 2009; Vytrasová et al., 2002). Leur présence n'est guère étonnante, compte tenu de leur prévalence élevée dans l'air extérieur et intérieur, ainsi que dans la farine de blé. Cette dernière peut constituer un réservoir de contamination dans l'environnement de production. Leur aptitude à tolérer des conditions de faible humidité, associée à leur comportement xérotolérant voire xérophile, favorise en outre leur prolifération dans les environnements de fabrication (Berghofer et al., 2003). En termes d'étude de la diversité interspécifique, il a donc pu être mis en évidence la contamination fongique au sein de l'usine, laquelle est non-négligeable dans les zones où la qualité de l'air n'est pas contrôlée, comme les zones avant cuisson et la zone de stockage intermédiaire. En revanche, les zones équipées de systèmes de filtration de l'air, telles que la sortie du four, présentaient des niveaux faibles de contamination démontrant l'intérêt majeur des méthodes préventives dans le contrôle des contaminations. Par ailleurs, le traitement des produits à l'aide de la solution d'éthanol permet une réduction significative de la fréquence d'altérations. Certaines études ont exploré la maîtrise de l'altération des produits de boulangerie en ajoutant des composés antifongiques au cours du processus de fabrication, tels que des composés phénoliques, des agents bioprotecteurs naturels comme la poudre de pelure de grenade, ou encore l'ajout de ferments pour leurs activités antifongiques (Borges Teixeira et al., 2024; Giri et al., 2024; Vytrasová et al., 2002). D'autres recommandations se concentrent davantage sur la composition des matrices alimentaires, le contrôle des points critiques, l'ajustement des conditions de cuisson et l'amélioration des stratégies d'emballage pour minimiser la contamination après cuisson et prolonger la durée de conservation (Chou et al., 2023; Debonne et al., 2018). En résumé, l'ajout de techniques de bioprotection et de contrôle pourrait être bénéfique, en complément d'un contrôle accru des mouvements de matériaux et de personnel au sein de l'usine. L'évaluation rapide de la diversité fongique et de sa répartition au sein de l'usine permet effectivement de formuler des recommandations plus ciblées faisant du MALDI-TOF une méthode toute désignée afin de répondre à la demande du marché industriel. L'étude de la diversité intra-spécifique des deux espèces dominantes, *C. sphaerospermum* et *P. brevicompactum*, a mis en évidence une diversité modérée au sein de l'usine, avec des isolats se regroupant en clusters génétiques distincts, susceptibles de représenter des populations

naturelles différenciées. La prédominance de certains haplotypes au sein de ces clusters, ainsi que leur présence accrue sur les cakes, suggère une persistance environnementale de ces haplotypes, entraînant des contaminations récurrentes à partir des zones post-cuisson. Ces haplotypes ne seraient pas de simples contaminants courants, mais pourraient être particulièrement adaptés aux niches écologiques spécifiques de l'usine et des cakes. Les schémas de contamination et les zones à risques ont été mis en lumière avec plus ou moins de précision. Toutefois, l'association de la spectrométrie MALDI-TOF avec le séquençage SSRseq a permis de mieux comprendre la diversité génétique et les sources de contamination de ces espèces. Certains haplotypes, en particulier ceux provenant des zones post-cuisson, étaient plus fréquemment retrouvés comme contaminants sur les gâteaux altérés, suggérant des événements de contamination répétés à partir de sources persistantes. Globalement, bien que la technique SSR soit pertinente et offre une discrimination suffisante pour le génotypage et source tracking, son coût, l'expertise nécessaire et le temps d'analyse rendent son application difficile dans un contexte industriel. En revanche, cette étude a démontré que la spectrométrie de masse MALDI-TOF bien que rapide, précise et moins coûteuse, ne permet pas encore de mettre en évidence les haplotypes et donc de réaliser un source-tracking précis. Une base de données contenant les spectres MALDI-TOF systématiquement associés aux données de génotypage SSR-seq, comme réalisé dans notre étude, mais appliquée à un très grand nombre d'isolats de l'espèce ciblée, permettrait (i) de mieux capturer la variabilité intra-espèce grâce aux données SSR-seq, facilitant ainsi la division en populations, sous-populations et haplotypes, et (ii) de mieux exploiter les spectres MALDI-TOF associés en optimisant l'identification des biomarqueurs spectrométriques spécifiques. Cela permettrait d'augmenter la capacité de discrimination intra-spécifique de la méthode MALDI-TOF, allant au-delà de la simple distinction entre clusters génétiques ou populations.

L'optimisation de l'exploitation des spectres MALDI-TOF pourrait notamment passer par l'exploration d'algorithmes d'analyse spectrale plus avancés, en particulier ceux basés sur l'intelligence artificielle et le machine learning, qui pourraient s'avérer particulièrement pertinents dans ce contexte (Feucherolles et al., 2021; Mohammad et al., 2023). Ces outils pourraient mieux discriminer les différences subtiles entre spectres et permettre une reconnaissance plus fine des haplotypes. Concernant l'accessibilité de l'analyse, il pourrait être intéressant de permettre aux industries de réaliser elles-mêmes les recalibrations rendant ainsi l'analyse plus accessible. Le typage par MALDI-TOF soulève cependant des questions méthodologiques importantes. Contrairement au typage génétique, qui repose sur des

marqueurs constants issus de l'ADN, non influencés par les conditions environnementales, les spectres obtenus par MALDI-TOF peuvent varier pour un même individu, compliquant ainsi l'identification de marqueurs spécifiques à une population. Cela rend impératif la standardisation des protocoles de culture et d'acquisition des spectres pour garantir une expression homogène des marqueurs discriminants. En conclusion, les résultats soulignent l'importance de stratégies ciblées de contrôle de la contamination, particulièrement dans les zones à haut risque. Les résultats de cette étude mettent en lumière la nécessité de mieux identifier les sources de contamination en milieu industriel. L'analyse a démontré le potentiel discriminant de la spectrométrie de masse MALDI-TOF, en particulier pour traiter des jeux de données volumineux.

Toutefois, il serait pertinent d'aller plus avant dans ce travail en entreprenant l'identification des biomarqueurs, c'est-à-dire identifier la nature des pics apparaissant sur les spectres MALDI-TOF et permettant la différenciation des espèces, des espèces au sein de complexes voire des populations ou des souches génétiquement différenciées. Afin d'identifier ces biomarqueurs, trois approches distinctes et complémentaires pourraient être utilisées. La première d'entre-elles consisterait à comparer directement des spectres et à identifier les pics discriminants par comparaison avec une base de données protéique générée *in silico* à partir des génomes des espèces étudiées. La seconde approche, dite « top-down », impliquerait l'extraction des protéines, suivie de leur analyse par MALDI-TOF tandem sans digestion préalable (Fagerquist, 2017). Les pics discriminants seraient ensuite identifiés par comparaison avec une base de données de spectres générée *in silico* grâce aux génomes des espèces d'intérêt. La dernière approche, dite « bottom-up », reposerait sur l'extraction des protéines totales des souches étudiées, leur digestion grâce à de la trypsine et leur analyse par nano-LC-MS/MS, avant identification par comparaison avec une base de données protéique *in silico*, basée sur les génomes des espèces analysées.

De manière plus générale, la spectrométrie de masse MALDI-TOF présente un potentiel considérable pour identifier tout composé ionisable, ouvrant ainsi la voie à de nombreuses autres applications. Parmi celles-ci, il pourrait donc être envisageable de développer une méthode de détection rapide et d'identification des mycotoxines dans les denrées alimentaires (Sivagnanam et al. 2017).

Valorisations scientifiques

Publications

Article et chapitre publiés

Rolland, N., Girard, V., Monnin, V., Arend, S., Perrin, G., Ballan, D., Beau, R., Collin, V., D'Arbaumont, M., Weill, A., Deniel, F., Tréguer, S., Pawtowski, A., Jany, J.-L., Mounier, J., 2024. Identification of Food Spoilage Fungi Using MALDI-TOF MS: Spectral Database Development and Application to Species Complex. *Journal of Fungi* 10, 456. <https://doi.org/10.3390/jof10070456>

Girard, V., Monnin, V., **Rolland, N.**, Mounier, J., Jany, J.-L., 2023. Identification of Mold Species and Species Complex from the Food Environment Using MALDI-TOF MS, in: *Microbiological Identification Using MALDI-TOF and Tandem Mass Spectrometry*. John Wiley & Sons, Ltd, pp. 497–513. <https://doi.org/10.1002/9781119814085.ch20>

Article à soumettre

Rolland, N., Mounier, J., Cadoret, M., Girard, V., Perrin, G., Deniel, F., Tréguer, Cloarec, O., Jany, J.-L. Diversity and source-tracking of spoilage molds in bakery products using MALDI-TOF MS and molecular approaches. Article à soumettre.

Contribution à d'autres articles

Destanque, A., Picot, A., Pensec, F., Pawtowski, A., **Rolland, N.**, Treguer-Fernandez, S., Debaets, S., Guérin, L., Coton, E., Hervé, M., Mercier, L. Contribution of grape berry mycobiota in fresh mushroom aroma production. Soumis à *ONEO One vine and wine*. 2024 (Contribution au traitement et à l'analyse des résultats dans le cadre de ce projet)

Ballan, D., Picot, A., **Rolland, N.**, Bovo, C., Prevost, C., Coton, E., Mounier, J. Diversity of spoilage microorganisms associated with fresh fruits and vegetables in French households. Article en préparation.

(Contribution au traitement et à l'analyse des résultats dans le cadre de ce projet)

Communications

Rolland, N., Girard, V., Monnin, V., Mounier, J., Jany, J.-L. Identification of Food Spoilage Fungi Using MALDI-TOF MS: Spectral Database Development and Application to Species Complex / Identification of Fungal Species and Species Complex from the Food Environment using MALDI-TOF MS. 22nd conference of Genomics and proteomics, Middlesex University, Londres, Angleterre, le 28 avril 2023 – **Présentation orale**

Rolland, N., Mounier, J., Jany, J.-L. Identification of Fungal Species and Species Complex from the Food Environment using MALDI-TOF MS. Journées Scientifiques (JS) de l'Ecole Doctorale Ecologie, Géosciences, Agronomie, Alimentation (ED EGAAL), Rennes, le 29 juin 2023 – **Présentation orale**

Rolland, N., Girard, V., Pages-Monteiro L., Monnin, V., Jany, J.-L., Mounier, J. Identification of Fungal Species and Species Complex from the Food Environment using MALDI-TOF MS. Microbres 2023, 18^{ème} congrès national de la Société Française de Microbiologie (SFM), Rennes, du 4 au 6 octobre 2023 - **Présentation poster**

Soumission d'abstract JS DE L'ED EGAAL – Microbes 2023

Identification of fungal species and species complex from the food environment using MALDI-TOF MS

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Abstract

Introduction and objectives

Fungi play a significant role in the global food losses and waste occurring each year due to their ability to colonize a very large diversity of food raw materials and processed foods along the food chain. *Aspergillus*, *Penicillium* and *Fusarium* are the main genera involved in food spoilage. Several species of these genera are known as mycotoxins producers which represent a major hazard for human and animal health. Rapid and accurate identification of fungi is therefore crucial to ensure food safety and quality. In recent years, MALDI-TOF MS has emerged as a valuable, rapid and reliable asset for microorganism identification. The aim of this study was to expand the future VITEK® MS IVD V3.4 spectral database representative of food spoilage fungi.

Material and methods

A total of 135 species including respectively 111 mold and 24 yeast species were selected including 6 species complexes corresponding to *Colletotrichum acutatum*, *Colletotrichum boniense*, *Colletotrichum gloeosporioides*, *Fusarium dimerum*, *Mucor circinelloides* complexes and *Aspergillus nigri* section. At least 2 strains and up to 10 strains with different isolations source were used for the spectral database building. For spectra acquisition, cultivation was performed on SDA, PDA, MEA, YGC, OGA media used in industrial microbiology and, concerning molds, spectra were acquired after two different growth times. The database performance was evaluated by cross-validation and external validation with more than 400 fungal isolates obtained in an industrial context.

Results, discussion and conclusion

Database performances were evaluated by cross-validation for 77 species included in the database and ~96% of correct identification to the species level was achieved. All species from the species complexes could be correctly identified (97% correct identification) showing the capacity of MALDI-TOF MS to differentiate closely-related species within species complex. The database was finally challenged in an industrial context with 75% of correctly identified strains showing the discriminating power of MALDI-TOF MS for the identification of fungal spoilers in the food industry.

Keywords

Fungi - Food spoilage - MALDI-TOF MS - Identification - Spectral database

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INTRODUCTION

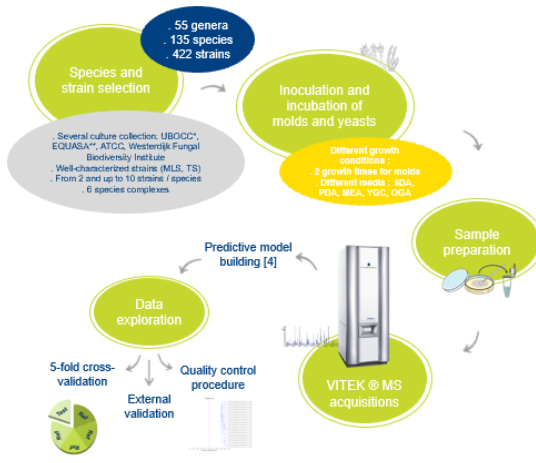
Fungi play a significant role in the global food losses and waste occurring each year. *Aspergillus*, *Penicillium* and *Fusarium* are the main genera involved in food spoilage. Several species of these genera are known as mycotoxins producers which represent a major hazard for human and animal health.

Rapid and accurate identification of fungi is therefore crucial to ensure food safety and quality. DNA barcoding is currently considered as the gold standard for fungal identification [1]. However, this technique requires expertise and can be tedious. In recent years, MALDI-TOF MS has emerged as a valuable, rapid and reliable asset for microorganism identification [2, 3].

The aim of this study was to expand the future VITEK® MS IVD V4.0 spectral database representative current concerns regarding food spoilage fungi as well as species of food technological interest with a specific emphasis on improving the efficiency of identifying species within species complexes as well as to evaluate its performances in an industrial context for the source tracking of spoilage molds.

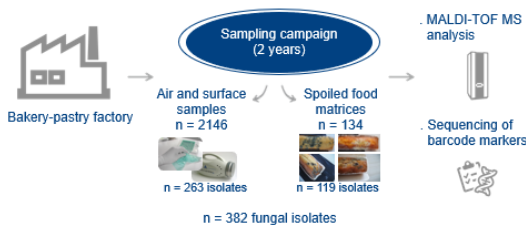
MATERIALS AND METHODS

1 Key steps of the spectral database extension and validation



* Université de Bretagne (Coastal Culture Collection (<http://uboccc.bio-qbrest.com>))
** Industrial strains collected over the years by the EQUASA technical platform (<http://chouaou.univ-brest.fr/equsa/equsa-coleforme-technologie/>)

2 External validation in an industrial context for source-tracking of spoilage molds



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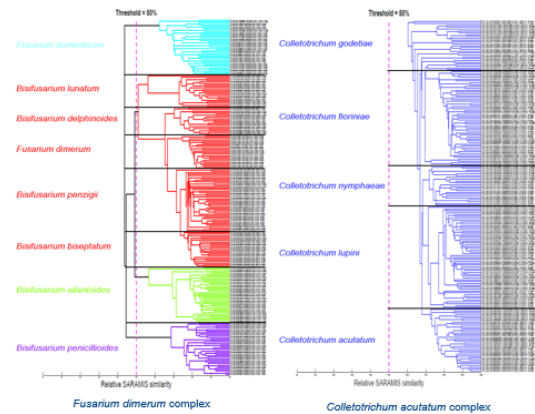
RESULTS

Cross-validation results

Cross-validation	Single choice ⁽¹⁾	No ID ⁽²⁾	Low discrimination ⁽³⁾	Overall result
Performance	91%	4%	5%	96%

(1) Correct identification – (2) Spectra not identified – (3) Multiple choice

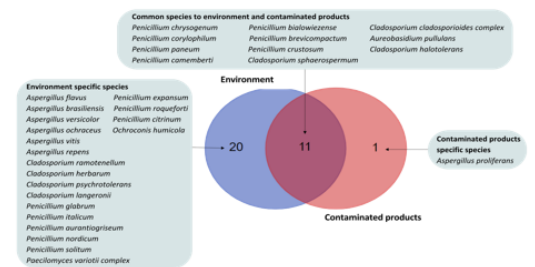
Efficiency of MALDI-TOF MS for discriminating species complexes



Differentiation of clusters for species within a same complex and clear species discrimination despite a high spectra similarity. *Fusarium dimerum* and *Colletotrichum acutatum* complexes have respectively 98% and 96% of correct identification to the species level.

Cross-validation results for all included species complexes range from 83% to 99%

External validation with a wide diversity of mold species with 11 species shared between environmental sources and spoiled products



72% of the isolates have been correctly identified to the species level (77% for the spoiled products and 68% for the environment).
15% of the remaining isolates belonged to species were not yet included in the database

CONCLUSION

- ~96% of correct identification to the species level was achieved and ~94% within species complexes using cross-validation approach
- The external validation of the database with independent isolates showed that MALDI-TOF MS can be used for source tracking of spoilage molds
- MALDI-TOF MS has enough discriminatory power to enable strain typing of food relevant species

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Titre : Identification et typage de levures et de champignons filamenteux d'intérêt alimentaire par spectrométrie de masse MALDI-TOF

Mots clés : Champignons- MALDI-TOF MS – Base de données - Complexes – Source-tracking

Résumé : Les champignons peuvent avoir des effets à la fois bénéfiques et indésirables sur les matrices alimentaires. Pour l'aspect positif, ils peuvent, par exemple, prolonger la durée de conservation et améliorer les propriétés organoleptiques. En revanche, ils sont des contributeurs majeurs aux pertes et gaspillages alimentaires mondiaux. Certaines espèces produisent des mycotoxines qui représentent une menace significative pour la santé humaine et animale. Il est donc crucial de maîtriser ces contaminants pour assurer la sécurité et la qualité des produits alimentaires ainsi que des environnements de production. L'identification précise des espèces fongiques est essentielle pour atteindre cet objectif. L'objectif de cette thèse de doctorat était d'élargir une base de données spectrales existante de MALDI-TOF MS avec des espèces et des complexes

d'espèces pertinents, ainsi que d'évaluer la diversité inter- et intra-spécifique dans un contexte industriel. À cette fin, un total de 380 souches de levures et de moisissures, représentant 133 espèces, ont été ajoutées à la base de données spectrale, incluant des espèces provenant de cinq complexes. La spectrométrie de masse MALDI-TOF a été employée avec succès pour évaluer la diversité fongique des moisissures environnementales et responsables de la détérioration des aliments dans une usine. Comparée à une méthode moléculaire, le séquençage SSRseq, la capacité de traçage des sources de contamination de la méthode MALDI-TOF MS a été confirmée. Elle a été appliquée spécifiquement à 66 isolats de *Cladosporium sphaerospermum* et 28 isolats de *Penicillium brevicompactum*, révélant respectivement quatre et trois populations distinctes.

Title: Identification and typing of yeasts and filamentous fungi from the food environment using MALDI-TOF MS

Keywords: Fungi - MALDI-TOF MS - Spectral database – Species complexes – Source-tracking

Abstract: Fungi can have both desirable and undesirable impacts on food matrices. On the positive side, they can, for instance, enhance shelf-life and improve organoleptic properties. Conversely, they are major contributors to global food losses and waste. Moreover, some species produce mycotoxins posing a significant threat to human and animal health. Controlling these contaminants is therefore crucial to ensuring the safety and quality of food products and production environments. Accurate identification of fungal species is key to achieving this. The aim of this PhD thesis was to expand an existing MALDI-TOF MS spectral database with relevant species and species complexes as well as to

evaluate the inter and intraspecific diversity in an industrial context. To this end, a total of 380 yeast and mold strains, representing 133 species, were added to the spectral database, including species from five complexes. MALDI-TOF MS was successfully employed to assess the fungal diversity of environmental and food spoilage molds in a factory. When compared to a molecular method, i.e. SSRseq, the source-tracking capability of MALDI-TOF MS was confirmed. Specifically, it was applied to 66 isolates of *Cladosporium sphaerospermum* and 28 isolates of *Penicillium brevicompactum*, revealing four and three distinct populations, respectively.