Instituto Superior de Engenharia
Politécnico de Coimbra

DEPARTMENT OF CHEMICAL AND BIOLOGICAL ENGINEERING

Exploring the fungal diversity in Portuguese honey

Exploração da diversidade fúngica no mel Português

Project Report to fulfill the Master's degree in Chemical and Biological Engineering

Author

Ivo Narciso Roxo

Student Number: 2013003311

FitoLab - Phytosanitary Laboratory, Instituto Pedro Nunes

Supervisors

João Trovão Lima Simões Antunes

António Luís Pereira do Amaral

Coimbra, Outubro de 2023

INSTITUTO POLITÉCNICO DE COIMBRA

INSTITUTO SUPERIOR DE ENGENHARIA DE COIMBRA

Abstract

Honey is a natural sweetener, produced by various species of bees. Due to its characteristics, honey is often considered an extreme environment. Nonetheless, honey is also known to contain diverse microbial communities, originating from the nectar, the bees and the environment. The microbial diversity in Portuguese raw monofloral honeys remains largely uncharacterized, constituting a serious gap in one of the country's most important industries. Mycological studies focusing on fungal species thriving on honey related products have a series of critical applications, ranging from the expansion of basic scientific knowledge, to the exploration of their industrial utilization, understanding their contributions to food spoilage and even environmental pathogen monitoring. The aim of this work was to provide a preliminary evaluation of the microbiological contamination of Portuguese monofloral honeys, particularly from local and commercial producers, from various botanical and geographic origins, and characterize the fungal diversity, using culture and non-culture dependent methods. The obtained results, by culture dependent methods, identified 18 fungal species, of which 15 are new records to the Portuguese honey fungi. The majority of the identified species came from the sample collected in the south region of Portugal (Region of Setúbal). In addition, taxa was detected that can potentially affect bee and human health, cause spoilage, and highlight bad bee-hive management practices. Through non-culture dependent methods it was possible to conclude that each honey harbors diverse and distinct microbiomes and the *Lavandula* spp. honeys had a higher diversity and evenness than the *Eucalyptus* spp. honey. Thus, the results obtained during the course of this work provide important information helpful to improve the quality and safety of this critical Portuguese product and industry.

Keywords: honey; fungal barcoding; fungal diversity; molecular biology.

Resumo

O mel é um alimento natural, produzido por diversas espécies de abelhas. Devido às suas características, o mel é muitas vezes considerado como um ambiente extremo. No entanto, é também um substrato conhecido por conter diversas comunidades microbianas, provenientes do néctar, das abelhas e do meio ambiente. A diversidade microbiana nos méis monoflorais portugueses encontra-se amplamente descaracterizada, constituindo uma grave lacuna numa das mais importantes indústrias do nosso país. Estudos micológicos com foco nas espécies de fungos que prosperam em produtos relacionados com o mel têm uma série de aplicações críticas que vão desde a expansão do conhecimento científico básico, à exploração da sua utilização industrial, à compreensão das suas contribuições para a deterioração de alimentos e até mesmo à monitorização de agentes patogénicos ambientais. O objetivo deste trabalho passou por realizar uma avaliação preliminar da contaminação microbiológica do mel monofloral português, particularmente de produtores locais e comerciais, com diferentes origens botânicas e geográficas, numa tentativa de caracterizar a diversidade fúngica, utilizando métodos quer dependentes quer não dependentes de cultura. Os resultados obtidos, por métodos dependentes de cultura, identificaram 18 espécies relacionadas com o mel monofloral português, das quais 15 são novas adições aos dados conhecidos relativamente a este substrato. A maioria das espécies identificadas provém da amostra recolhida na região sul de Portugal (Região de Setúbal). Além disso, foram detetados táxons que podem potencialmente afetar a saúde humana e serem prejudiciais para as abelhas, apontando assim para más práticas de manutenção das colmeias. Através de métodos não dependentes de cultura, foi possível verificar que cada mel apresenta diversos e distintos microbiomas e que os méis de *Lavandula* spp. apresentavam uma maior diversidade, quando comparados com o mel de *Eucalyptus* spp. Desta forma, os resultados obtidos ao longo deste trabalho fornecem informações importantes que podem ser úteis para melhorar a qualidade e segurança deste importante produto ao nível da indústria portuguesa.

Palavras-chave: mel; barcoding fungos; diversidade fúngica; biologia molecular.

Acknowledgements

First of all, I want to thank my family, and especially my sister Isa Narciso Roxo, my father Joaquim Jorge das Neves Roxo and my mother Elisa Rosa Narciso and my grandparents, for being my source of inspiration, for having always supported in everything, for believing in me and above all for the patience they had throughout this stage. Without your help I would never have been able to achieve what I have achieved until today and I owe you a lot.

I would like to thank Doctor João Trovão Lima Simões Antunes from FitoLab - Phytosanitary Laboratory and Professor Doctor Antonio Luís Pereira do Amaral from ISEC - Coimbra College of Engineering, for having agreed to be my supervisors, for all their help and availability in contributing ideas, sharing knowledge and learning and for answering all kinds of questions regarding this thesis.

I would like to thank Professor Doctor António Manuel Santos Carriço Portugal and Professor Doctor Joana Cardoso da Costa, directors of FitoLab - Phytosanitary Laboratory, for all their support, patience and availability during this work.

I also want to thank my friends for the advices and for all the support and friendship shared over the last year.

Thanks to all the people who along this path have provided me with knowledge both at work and personally.

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List of acronyms

a_W	Water activity
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DG18	Dicloran 18% Glycerol Agar
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
FB1	Fumonisin B1
HTS	High-Throughput Sequencing
ITS	Internal Transcribed Spacer
LPCB	Lactophenol Cotton Blue
LSU	Large Subunit
NGS	Next-Generation Sequencing
OTA	Ochratoxin A
OTU	Operational Taxonomic Unit
PAT	Patulin
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid

List of Chemical Compounds

CO_2	Carbon dioxide
H_2O_2	Hydrogen peroxide

1 INTRODUCTION

1.1 Foodstuffs fungal diversity and spoilage – general considerations

1.1.1 Fungi and food spoilage

Foods are organic materials that are consumed for their nutritional value. They come from plants, animals or fungi and include organic compounds such as proteins, fats, carbohydrates and minerals. Food spoilage is defined as the metabolic processes leading to the transformation of foodstuffs into an unacceptable form for human consumption (through microbiological, chemical and/or physical action) (Figure 1). Food spoilage is thus related to food safety (Boylston, 2005). Different physical, microbiological, or chemical actions and agents can cause food spoilage (Amit et al., 2017). Understandably, this poses a major challenge for humankind. Today, up to one-third of all food is spoiled or wasted before it is eaten. This equates to approximately 1,3 billion tons per year (FAO, 2011; Garnier et al., 2017). Common agents of foodstuff spoilage are parasites, bacteria and fungi. Being ubiquitous in nature and producing various mycotoxins, fungi are common in foodstuffs and pose a serious threat to both human and animal health (Garnier et al., 2017; Shephard, 2008). They are particularly problematic, since they are disseminated through spores released into the environment. Air can be a vector for disseminating almost all fungi significant to food spoilage. However additional factors related to food production can also be a source of fungal contamination (Dao & Dantigny, 2011; Scholte, 1995).

Every manufactured food has a set of standard characteristics that set it apart from the remaining, such as its consistency, odor, taste, and scent. Any modification to these characteristics renders the food product to be considered suspect or unacceptable (Abdullahi et al., 2019). When detrimental bacteria and fungi are present in food, microbial food deterioration may occur, making the food product unappealing or potentially harmful (Abdullahi et al., 2019; Petruzzi et al., 2017; Houghton, 2016). Unlike bacteria, fungi can quickly invade tissues and develop in the substratum (Dao & Dantigny, 2011). For science and industries looking for effective ways to prevent and/or limit fungal growth or development in food products, control of fungal spoilage is a crucial challenge (Ross et al., 2002). The ability of fungi to use a variety of substrates, such as carbohydrates, organic compounds, proteins, and lipids, may help to explain their astounding capacity for adaptation. Additionally, these fungi may withstand chemical and/or physical preservatives that are occasionally used to extend the shelf life of products, as they are often considered acidotolerant, xerotolerant, and/or psychrotolerant (Garnier et al., 2017; Huis in't Veld, 1996).

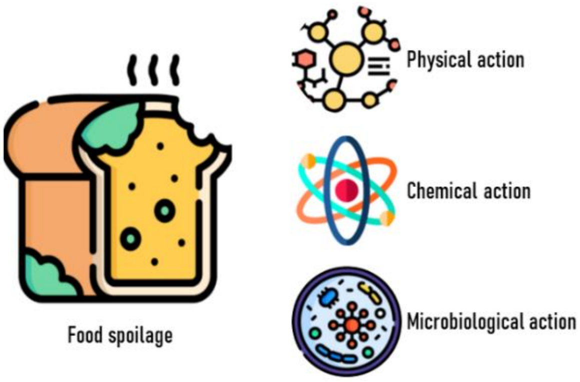


Figure 1.1 – Causes of food spoilage (constructed with icons from flaticon (https://www.flaticon.com/)).

1.1.2 Food spoilage fungal species

Molds (filamentous fungi), a class of fungi that generates multicellular filaments known as hyphae as they grow, pose a serious threat to the food industry, given that they are prolific food spoilage organisms. Most fungal species are saprophytic and have adapted to obtain nutrients from inanimate organic matter (Snyder et al., 2019). Several fungal species are airborne dispersed but can also be present in the raw materials themselves. However, only a small number of species are to blame for a particular food product's fungal deterioration (Filtenborg et al., 1996). The associated mycobiota of a given food product is, usually, constituted by less than ten species (Shapaval et al., 2013). Therefore, and taking this information into account, the handling of undesirable molds in the food industry is commonly focused on the specific associated mycobiota of the relevant product (Pitt & Hocking, 2009).

Many important food spoilage fungi can be described as "moderate xerophiles", such as species in the genera *Penicillium* and *Aspergillus* (Pettersson & Leong, 2011). Almost all xerophilic fungi (i.e., capable of growth in water activities, a_W, below to 0.85) are ascomycetes that can produce ascospores in asci. The most frequent ascomycete species responsible for dried cereal deterioration are those belonging to the genus *Aspergillus* (former *Eurotium*). *Neosartorya*, *Talaromyces*, and other ascomycetes species (such as *Byssochlamys* species and *Eupenicillium lapidosum*) produce ascospores with extremely high heat resistance that can survive heat processing and are in charge of causing food spoilage after pasteurization (Dao & Dantigny, 2011). For the food industry, these species have an economic impact since they can grow on stored grain, cereals, or preserved foods with high sugar (e.g., jams or maple syrup) or salt contents (e.g., biltong or dried fish) (Kačániová et al., 2009; Pitt & Hocking, 2009; Samson et al., 2010).

Spoilage fungi are mainly associated with the Ascomycota and Mucoromycota phyla. Moreover, many zygomycetes, *Penicillium* and *Aspergillus* species are deeply related to food spoilage (Pitt & Hocking, 1997). Zygomycetes are widespread in nature and are thought to be relatively basic fungi. They develop quickly on simple carbon sources found in plant debris and soil, and their spores are frequently found in indoor air.

Mucor and *Rhizopus* are the two more often reported species causing food spoilage. Although zygomycetes aren't recognized for producing mycotoxins, several species have been reported to produce other harmful substances. On the other hand, *Penicillium* is commonly found in soils and plant waste. Their reproductive features, which generate chains of conidia, can set them apart from other pests that ruin fruits and vegetables. In comparison to *Penicillium*, *Aspergillus* often develop more quickly and are more resistant to high temperatures and low a_W. Several *Aspergilli* species produce mycotoxins, including aflatoxins, ochratoxin, territrems, and cyclopiazonic acid, and they can contaminate a wide range of foods and non-food products (Doyle, 2007; Pitt & Hocking, 2009). The fact that *Penicillium* and *Aspergillus* are significant food spoilage species, as well as common indoor fungi, shows that their spores play a significant role across the food production chain (Dijksterhuis, 2017).

1.1.3 Food spoilage economic impacts

Fungal spoilage causes great economic losses worldwide (Dantigny et al., 2005). Despite improved industrial standards, fungi still seriously threaten food safety. In fact, these microorganisms can develop at the field level, but can also grow while raw materials are being stored, transported, and sold, which results in significant yearly financial losses (Dao & Dantigny, 2011). Given their wide variety, fungal spoilage causes significant financial losses throughout the food chain, affecting producers (e.g. farmers), retailers and consumers (Odeyemi et al., 2020; Snyder & Worobo, 2018). Farmers revenues and consumers expenses is directly and negatively impacted by avoidable food spoilage (Rawat, 2015). In fact, fungi are thought to be responsible for approximately 5% to 10% of the world's food production losses (Garnier et al., 2017; Pitt & Hocking, 2009). However, it is very difficult to assess losses attributable to molds (Dao & Dantigny, 2011). For example, depending on the season, the type of product, and the processing method, these losses can vary from 1% to 3% of the products in the bakery industry, with estimates often pointing to losses up to 5% (Dantigny et al., 2005). On the other hand, nearly 20% of food losses occurs in fresh products and fresh milk, with lesser amounts coming from grain products (15,2%), caloric sweeteners (12,4%), processed fruits and vegetables (8,6%), meat, poultry, and fish (8,5%); and fats and oils (7,9%) (Sahu & Bala, 2017).

The implications of food losses and waste are broad and are directly connected to food and nutritional security, environmental health and economic losses (Gunders, 2017; Martin et al., 2021; Papargyropoulou et al., 2014). In addition, food losses result in waste of land, water, energy, and resources used in their production. Moreover, wasted food products result in additional carbon dioxide (CO_2) emissions as well as a decline in the food's economic worth (Rawat, 2015). However, the most important aspect of mold spoilage of food is the formation of mycotoxins (Filtenborg et al., 1996).

1.1.4 Mycotoxins and related problems

The most dangerous chemical substances reported in food and feed products are mycotoxins, which constitute the largest risk to both human and animal health (Iqbal et al., 2014; Misihairabgwi et al., 2019). Indeed, mycotoxins are considered a major threat to human and animal health, especially in developing countries, as they cause a range of adverse health effects (Shephard, 2008). Such mycotoxins refer, broadly, to a set of harmful toxins that molds create. However, the toxicity of the produced metabolites greatly varies, with chronic intoxications being the most significant for humans (Filtenborg et al., 1996). Indeed, numerous pathological problems in humans are thought to be caused by mycotoxins produced when mold grows on food. Additionally, through the consumption of meat from animals fed with contaminated diets, and the use of contaminated spices (in meat processing), mycotoxins can also be transferred to humans. Some mycotoxins are found only in molds, but most of them are excreted into food (Filtenborg et al., 1996). Since most mycotoxins are highly resistant to physical and chemical treatments, the rule of thumb is that, once mycotoxins are in food, they will remain there during processing and storage (Scott, 1991).

A given species can produce different types of mycotoxins, and a given foodstuff can be contaminated by various mycotoxins (Ndiaye et al., 2022). This plays an important ecological role in the diversification of these microorganisms and their adaptation to cultivated plants for food and feed production (Lima & Santos, 2017). Toxin producing fungi in food are classified into field fungi and storage fungi based on their ecological requirements for growth (Adeyeye, 2016; Bayman & Baker, 2006). The most frequent microbial contaminants affecting crops during the pre- and post-harvest periods are saprophytic molds (*Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, and *Claviceps*) (El-Sayed et al., 2022).

Furthermore, the most prevalent mycotoxins that can contaminate food and feed include aflatoxins, ochratoxin A, zearalenone, deoxynivalenol, nivalenol, fumonisin B1 and B2, and patulin (Ndiaye et al., 2022; Siri-anusornsak et al., 2022). *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nominus* and *Aspergillus niger* are the primary producers of aflatoxins, which are secondary fungal metabolites (Majer-Baranyi et al., 2016; Xie et al., 2017; Zhao et al., 2019). A mycotoxin known as patulin (PAT) is mostly produced by *Penicillium* (Rodríguez-Bencomo et al., 2020), *Byssochlamys* and *Aspergillus* species (Li et al., 2019). Deoxynivalenol (DON) and zearalenone are β-resorcylic acid lactones (Frizzell et al., 2015), mainly produced by *Fusarium* species (Bönnighausen et al., 2019). Ochratoxin A (OTA) is mostly produced by *Penicillium verrucosum* and *Aspergillus ochraceus* (Abarca et al., 2019), however, other species such as *Aspergillus niger* and *Aspergillus carbonarius* can also produce it. Fumonisin B1 (FB1) and trichothecene mycotoxins produced by *Fusarium* species such as *Fusarium verticilloides* and *Fusarium proliferatum* are also known to be problematic, being associated with diverse toxic effects (Arumugam et al., 2019; Ndiaye et al., 2022).

Mycotoxin contamination is thought to be the source of large economic losses, as well as concern with food safety and public health. However, their full impacts are impossible to measure. Every year, mycotoxins affect 25% of the harvested crops, resulting in enormous economic losses for the agricultural and industrial sectors (El-Sayed et al., 2022). It may never be possible to determine the precise sum of global economic losses brought in by mycotoxin contamination. In addition to the obvious losses from food and feed spoilage, there are also problems arising from: lower productivity, foreign exchange earnings, costs for inspection during shipment, compensations paid in case of claims, farmer subsidies, costs for research, training, extension program and detoxification. These charges could be astronomical when added together (Adeyeye, 2016; Coulibaly, 1989).

1.2 Beekeeping – Social and Economic Impacts

1.2.1 Social impacts

Beekeeping (or apiculture) is a zootechnical activity centered on the upkeep of honey bee colonies, typically in hives, with the goal of sustaining and exploiting the domestic bee *Apis mellifera* economically and rationally, in order to gain access to their direct goods and by-products. Assets such as honey, propolis, pollens, royal jelly, bee bread, waxes and apitoxins (bee venoms), have a wide and diverse range of applications, including in the cosmetic, food, pharmacological and therapeutical industries (Pasupuleti et al., 2017). This activity, which brings multiple benefits to society (Aryal et al., 2020; Gelats et al., 2017), contributes to the sustainable development of rural areas (Hanley et al., 2015; Klein et al., 2003) and helps develop global sustainability (Etxegarai-Legarreta & Sanchez-Famoso, 2022; Vrabcová & Hájek, 2020; Wood et al., 2018). This is noted, for instance, in the expansion of apitherapy (an alternative therapy using products coming directly from honey bees) in Western culture in the last few years (Pereira, 2016). As the oldest sweetener used by humans, honey is valued globally, embraced by religious and cultural beliefs, and is now recognized as a potential aid in the treatment of cancer as well as a homeopathic treatment option for wounds, burns, oral health and other ailments (LayFlurrie, 2008; Bardy et al., 2008). Moreover, beekeeping is also considered a valuable and good example of an environmentally sustainable production system, with notorious positive impacts on the global biodiversity and agriculture. Other ecosystem benefits provided by honey bees, comprise several regulating, provisioning and cultural functions related to the human well-being and development (Aryal et al., 2020). Overall, this activity is mainly characterized by three advantageous outcomes, namely: (1) a confluence of economic interests (by the production of honey and by-products of the hive, that can provide remunerative gains); (2) social impacts (since it contributes to the establishment of the rural population in territories where other economic activities are hard to be developed or maintained); and (3) a contribution to environmental maintenance, sustainability and health (through pollination of cultivated and wild fields) (Figure 1.2) (Estevinho et al., 2012; Etxegarai-Legarreta & Sanchez-Famoso, 2022).

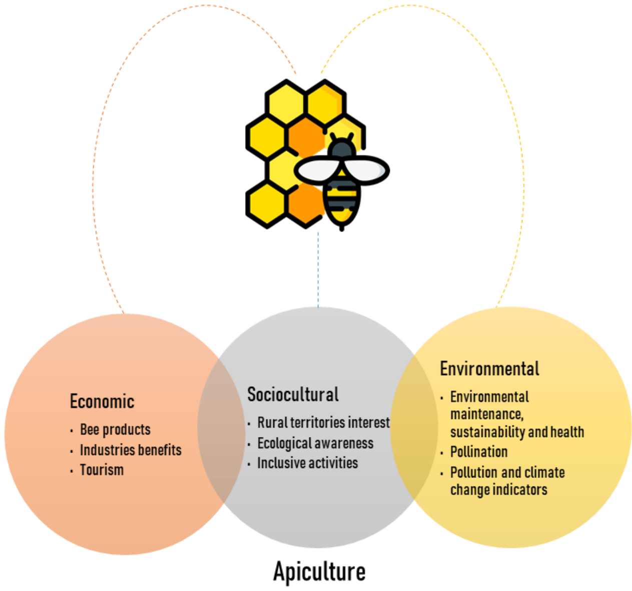


Figure 1.2 – Beekeeping main advantageous impacts (constructed with icons from flaticon (https://www.flaticon.com/)).

1.2.2 Economic impacts

World honey production has fluctuated substantially over the past few years, partly due to the occurrence of various economic crises and more recently due to the COVID-19 pandemic. However, such factors did not prevent the continuous registration of a growing trend, with the most recent data made available by the Food and Agriculture Organization (FAO, 2020), revealing that almost 390 thousand tons of honey were produced solely in Europe. According to this report, Portugal had a production of approximately 10 thousand tons, a value relatively distant from countries such as Ukraine (68 thousand tons), Russia (66 thousand tons) or Spain (30 thousand tons) and below the European average (23,5 thousand tons) (FAO, 2020). Nonetheless, honey production is still a critical industry in multiple regions of the country, contributing strongly to their economy and sociocultural activities (e.g., Lousã, Castelo Branco and Serra da Estrela). On the other hand, the analysis of the data regarding the evolution of honey production and consumption in Portugal, also revealed a decrease in honey production before 2018, contrasting with the increase in consumption and demand in the same time period (FAO, 2020). Nonetheless, the data also shows that in the last 5 years, an annual growth of 3,44% has occurred, reflecting a strong investment, the commitment towards the continuous improvement of production conditions within this field, and the promotion and valorization of these products (FAO, 2020; INE, 2018). These results reflect both the increase of the products quality and the measures implemented to reach increasingly higher levels of demand, while also revealing the importance of the sector and its impact on the country's economy.

1.3 Beekeeping – Environmental and Food Safety Impacts

1.3.1 Environmental impacts

Honey bees are considered a keystone species and a major contributor to the ecosystem. This is mainly due to their vast contribution to pollination activities (approximately in 70-80% of crops worldwide), food production, biological control of ecosystems and upkeep of biodiversity (Krishnan et al., 2020), and having a role as bioindicators for disturbances in ecosystems and in the environment (Famuyide et al., 2014; Pérez-Piñeiro, 2017). As forests are important sources of forage for bees (Girma & Gardebroek, 2015; Degu &Megerssa, 2020; Ricketts & Shackleton, 2020; Wagner et al., 2019), this activity could also support forest conservation. The therapeutic qualities and health advantages of beekeeping products, as well as their role in nutrition, human health, and food security, have been the subject of recent discoveries (Abro et al., 2022; Manyi-Loh et al., 2011; Smith et al., 2015; Teklewold et al., 2021). Moreover, the pollination activity of honey bees, also enhance crop reproduction and ultimately increase the quality and quantity of food items (Aryal et al., 2020; Kačániová et al., 2012).

1.3.2 Food Safety impacts

New technologies and innovative uses of honey and bee-associated products are currently expanding marketing opportunities, with the quality of honey required to be determined by, mainly, its sensorial, chemical, physical and microbiological characteristics. The microbiological characteristics of honey are inherent to quality and safety and need to be associated with these opportunities. However, the safety of the honey products is yet to be sufficiently assessed (Aryal et al., 2020; Kashyap et al., 2019; Martins et al., 2003). For this purpose, a comprehensive understanding of their microbiological characteristics has to be secured, in order to ensure the production of a quality and safe final product (Kacániová et al., 2009).

From a microbiological perspective, honey can have two sources of contamination by microorganisms: (1) primary sources: including pollen, the digestive tracts of honey bees, dust, air, soil and nectar (more difficult to control); and (2) secondary sources: arising from human honey manipulation, while also including risks related to air quality, food handlers, cross-contamination and the state of equipment and buildings used in the process (more easily controlled by good manufacturing practices) (Felsöciová et al., 2012). Residues and contaminants are also important issues in the field of food safety, since their presence can lead to negative impacts on human health (Kellmann et al., 2009). Consequently, legislation has been established at both the national and international levels (although scarcely) to protect consumers from exposure to food contaminants and toxins, being required for the monitoring and control of such compounds throughout the food chain. The number of surveyed contaminants, and the demand for their analysis, continues to grow, mainly due to the identification of new toxins and an increasing consumer awareness, which ends up creating the need for the development of new control measures. Nonetheless, while the European Commission proposes maximum concentration limits for mycotoxins in various types of foods, they are often lacking or incomplete when considering bee products (Kostić et al., 2019).

Honey spoilage is not an often-reported phenomenon, mainly due to their associated antimicrobial properties. The mechanism of the antimicrobial action of honey are most likely a combination of several different factors. Its low water activity is likely to play a major role when honey is applied topically, although studies comparing its effect to sugar syrups of the same concentration have shown that honey has superior activity (Medicines, 1998). The hydrogen peroxide (H_2O_2) produced enzymatically in honey is also considered one of the main reasons for such biological activities (Carvalho et al., 2010). This occurs through the action of the bee-derived enzyme glucose oxidase, which transforms glucose into gluconic acid and H_2O_2, along with several non-peroxide-dependent mechanisms that can add additional beneficial substances to honey (Grabowski & Klein, 2015). Moreover, this effect is more accentuated in honeys with high pH and moisture content, due to the presence of glucose oxidase, and the formation of fewer amounts of gluconic acid at low pH. These reactions are also strongly dependent on the floral source (whose chemical compounds may increase or decrease this activity) and the processing and eventual dilution of the honey (Bang et al., 2003; Basson & Grobler, 2008; Chen et al., 2012; Snowdon & Cliver, 1996). In addition, the floral source itself may also be a critical factor, being dependent for instance, on the season, plant type and location (Carvalho et al., 2010; Grabowski & Klein, 2015; Sinacori et al., 2013). In fact, a low pH of the honey (average pH is around 4) provides the acidity necessary to avoid spoiling in most of the cases. This acidity is a result of various acids, mainly gluconic, but also formic and citric, produced by the action of bee enzymes on some of the honey glucose molecules (Interest, 2014).

Honey chemical substances and other physicochemical properties, like low redox potential or high viscosity often resulting from bee activities, are also reasons why mature honey is relatively resistant towards microbiological contamination (Grabowski & Klein, 2015). Moreover, an important physicochemical property that can affect the development of microorganisms in honey is a_W (water activity). This parameter is a measure of the amount of water in a substance available to support microbial growth and varies on a scale of 0 to 1 (Interest, 2014). According to the currently available literature, honey is characterized by a low a_W, between 0.593 and 0.637, which inhibits the growth of almost all microorganisms (Snowdon & Cliver, 1996). However, if the moisture content is high (above 21%), fungi and yeasts can develop and cause honey fermentation. This implies that the product stability will depend on the microbial content and on the ability of osmophile and xerotolerant/xerophile organisms to grow (Rodríguez-Andrade et al., 2019; Snowdon & Cliver, 1996). The natural acidity of this product, coupled with a low protein content, high osmolarity, low water activity, high viscosity and the unfavorable conditions associated with the penetration of atmospheric oxygen are particularly stressful for several microorganisms, since they create an adverse environment for their development. However, since bees collect pollens/nectars, yeast and fungi presence in honey is unavoidable (Felsöciová et al., 2012; Sinacori et al., 2013). As such, and despite its chemical properties and their particular physicochemical nature and biological origin, honey contains a rich microbial population and can be an ideal substratum for the development of particularly resistant microorganisms like xerotolerant and xerophilic fungi (Martins et al., 2003; Rodríguez-Andrade et al., 2019; Sinacori et al., 2013).

1.4 Honey – Composition and Characteristics

1.4.1 Honey's chemical and physical composition

According to the European Union Legislation (DLgs 179/2004) and the *Codex Alimentarius* (CODEX STAN 12-1981), honey is a natural sweetener produced by honey bees from: (1) flower nectars (blossom honey or nectar honey); or (2) carbohydrate-rich secretions of plants or even excretions of plant-sucking phytophagous aphids (honeydew honey), after combination with the animal specific molecules, placement, dehydration, and storage in the honeycomb (to ripen and mature) (Carvalho et al., 2010; Rodríguez-Andrade et al., 2019; Sinacori et al., 2013). Honey composition is complex and variable, and it contains at least 181 different substances (Albaridi, 2019; Alvarez-Suarez et al., 2010). It's mainly composed by sugars turned into a super saturated solution containing mainly the monosaccharides fructose and glucose (in a concentration not lower than 60%) and by a much lesser amount of oligosaccharides, organic acids, enzymes (amylases and α-glucosidase) and colloids (Rodríguez-Andrade et al., 2019). Honey has been reported to also contain a variety of flavonoids and phenolic acids, which have extensive biological properties and are responsible for their antioxidant and anti-inflammatory effects (Cianciosi et al., 2018; Da Silva et al., 2016; Ferreres et al., 1994; Matović et al., 2018; Soares et al., 2017). Whilst nectar composition varies from plant to plant, and a range of other chemical compounds are commonly present, often the dominant and primary sugar is sucrose. Single-source honey has a narrower range of values due to higher consistency in nectar composition (Ball, 2007). The varying concentrations of the different nectar components are therefore, the reason why a wide range of distinct types of honey are available, since they are mainly a result of the type of nectar the bees predominantly collected (Interest, 2014). Thus, the exact composition varies tremendously, depending on the plant species and environmental conditions, having consequently, a definite impact on the physical characteristics and quality of the mature honey (Ball, 2007).

The composition, color, aroma and flavor of honey, depends mainly on the flowers, geographical region, climate, and the honey bee species involved in its production. However, seasonal differences can also be important and affect the composition of honey (Molan, 1992). In some cases, it can also be affected by weather conditions, processing, manipulation methodologies, packaging and storage time (Da Silva et al., 2016). The honey color is also variable, depending on its composition and reflecting the various components present such as polyphenols, minerals, and pollen (Gomes et al., 2010). It is known that the darker honey is richer in minerals, has a higher amount of pigments such as flavonoids (Taormina et al., 2001) and has a stronger flavor, while the lighter (sometimes even colorless) is poorer in terms of mineral salt contents (Escuredo et al., 2013; Karabagias et al., 2014). In addition, the color is also directly related to the origin of the nectar and to the bee species involved in its production. Honey composition results from two main reactions from the nectar: (1) the dehydration process; and (2) the transformation of sucrose into glucose and fructose, through the enzyme invertase (Marassi, 2010). However, the full characterization of honey is not common and there is still a lack of information about the characteristics of various organic certified honeys (Estevinho et al., 2012; Magyar et al., 2016).

Depending on the geographical origin of production, honey may be associated with specific regions within the European Union (EU) under the Protected Designation of Origin (PDO) and Protected Geographical Identification (PGI) labels. Honeys bearing these labels generally exhibit properties that are inherently or exclusively associated with a particular region or local environment with inherent natural and human factors (Development, 2019). Portugal currently has the highest number of honeys registered in the EU (9 PDO honeys), followed by Spain (5 PDO honeys and 1 PGI honey) and France (2 PDO honeys and 3 PGI honeys) (Development, 2019; Machado et al., 2020).

1.4.2 Multifloral and monofloral honey characteristics

Organic honey production is an ecologically based system, which encourages the use of good agricultural practices to maintain the agricultural ecosystem balance and diversity, while also promoting the sustainable use of natural resources, environmental quality, animal welfare and human health. Literature points out that the botanical origin of honey, beehive type, and material, have an influence on the development of bee diseases and the quality of honey (Estevinho et al., 2012). According to the botanical origin, floral honeys are classified as poly (or multifloral) and mono (or unifloral) (Bobis et al., 2020; Directive, 2002). This type of honey, when compared with others (e.g., honeydew honey), has a lesser mineral and oligosaccharide content. Multifloral honeys derived from multiple botanical sources are the most commercially available (Alvarez-Suarez et al., 2010; Directive, 2002; Seraglio et al., 2021). Nonetheless, the progressive increase in the market of imported honey, with lower prices and inferior quality, has recently led to a growing need to assess the local authenticity, especially for the case of monofloral honeys (using a full quality control based on a physicochemical, microbiological and geographical description). In this type of honey, the pollen spectrum is based on a single species holding more than 45% of the pollen, with the exception of monofloral rosemary (*Lavandula* spp.) honey, which has pollen percentages of the respective pollen type greater than 10%. In addition, the monofloral honeys typically offer a unique sensory experience, and in certain cases are associated with therapeutic effects according to their botanical origin (Alvarez-Suarez et al., 2010; Directive, 2002; Seraglio et al., 2021). One example is eucalyptus honey, which has become a popular single flower honey among consumers due to its aroma and botanical medicinal properties (Barbosa et al., 2016; Bobis et al., 2020). However, the most widely available honey in the market is blended honey, consisting of a homogenous mixture of two or more honeys with different characteristics such as geographical origin, botanical source, color, flavor, or density (Baroni et al., 2006; Bird et al., 2013; Machado et al., 2020).

1.4.3 Honey mycobiome

Due to their natural properties, and sometimes to the general control measures applied in the industry, honey is a product with a low diversity and minimal levels of microbes (Carvalho et al., 2010). Nonetheless, the microbes of concern/interest in honey are primarily yeasts, fungi and spore-forming bacteria, since their presence can sometimes influence the product stability and its hygienic quality (leading to spoilage). These microorganisms may be involved in activities such as spoilage, production of enzymes, antibiotics, mycotoxins and growth factors (vitamins, amino acids), metabolic conversion of provisions, and inhibition of competing microorganisms. Examples of filamentous fungi usually found in honey are *Aspergillus, Penicillium*, *Mucor* and *Monascus,* along with some osmophilic yeasts such as *Saccharomyces* (Felsöciová et al., 2012; Martins et al., 2003).

Typically, fungi are organisms that grow as filaments, termed hyphae, which extend only at their extreme tips. Fungal hyphae branch repeatedly behind their tips, giving rise to a network termed a mycelium, in contrast to many other filamentous organisms which grow by repeated cell divisions within a chain of cells (intercalary growth) (Deacon, 2006; Kashyap et al., 2019). Filamentous fungi are often considered as the more ubiquitously found in nature and often have thermal resistant spores, with a great capacity of surviving in various environmental conditions. They can be introduced in honey by man, through dust, water installations or containers, or even by the bees themselves through pollen collection (Kačániová et al., 2012). Due to the particular physicochemical nature of honey, certain types of microorganisms such as xerophilic fungi are key contaminants, that may emerge due to their ability to grow under conditions of reduced a_W (since they can complete their life cycle on substrates that have been dried, concentrated or hold the presence of high levels of soluble salts or sugars) (Pitt & Hocking, 2009). Some authors have used the term "xerotolerant" rather than "xerophile", arguing that these fungi exhibit "tolerance for" rather than "love for" reduced a_W (Pitt & Hocking, 2009). In fact, xerotolerant is an inappropriate term for extremely xerophilic fungi such as *Aspergillus penicillioides, Aspergillus halophilicus, Basipetospora halophile*, *Chrysosporium fastidium* and *Xeromyces bisporus*, which have an absolute requirement for reduced a_W (Kanekar & Kanekar, 2022; Pitt & Hocking, 2009). Their survival in nature depends on access to environments of reduced a_W where competition is restricted or eliminated (Pitt & Hocking, 2009). Although many xerophiles grow optimally at quite high a_W, most are basically slowly growing fungi and cannot compete in mixed cultures at such levels with the ones that prevail in soils or fresh foods (Kanekar & Kanekar, 2022; Pitt & Hocking, 2009). This work follows the definition of Pitt (1975) where a xerophile is a fungus capable of growth, under at least one set of conditions, at an a_W below 0.85.

The common obligate xerophiles *Ascosphaera apis* and *Bettsia alvei*, indicators of bad bee-hive management practices (Felsöciová et al., 2012; Migdał et al., 2000) have been reported in honey, as well as several xerotolerant species of *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* (e.g. Rodríguez-Andrade et al., 2019). On the other hand, strains of *Saccharomyces*, *Schizosaccharomyces* and *Torula* predominate among yeasts (Migdał et al., 2000). Moreover, additional studies also report the presence of several plant pathogenic fungi (Seijo et al., 2011). In addition, the presence of some mycotoxin-producing *Aspergillus* species, such as *Aspergillus fumigatus* and *Aspergillus niger*, has also been reported (Kačániová et al., 2012). Ecologically, studies in Slovak honey also revealed that the primary sources of the microbial community present in honeys originate from pollens and the microorganisms present in the digestive tract of bees, which mainly comprise microorganisms normally present in dust, air and flowers (Kacániová et al., 2009). Moreover, knowing that fungi frequently live in a strong association with plants, different fungal species assemblages might be as typical of the honey as the spectrum of pollen grains. In fact, honeys are also rich in fungal spores, and the diversity of fungal species associated with these conditions makes this an important subject of study based on the ecological, phytopathological, and pharmaceutical impacts (Magyar et al., 2016). So far, more than 120 fungal species have been isolated and identified from honey samples worldwide (Checklist 1.1). Moreover, if identifications conducted solely through direct spore morphology are considered, this number greatly expands (Pérez-Atanes et al., 2001; Diez et al., 2004; Dimou et al., 2006; Carmen Seijo et al., 2011; Olga et al., 2012; Magyar et al., 2005, 2016; Mura-Mészáros & Magyar, 2017).

Checklist 1.1 – Checklist of fungi isolated and identified from honey, honey blossom and honeydew between 2000 and 2022*.

Phylum *Ascomycota* Caval.-Sm.

Class *Dothideomycetes* O.E. Erikss. & Winka

Order *Capnodiales* Woron.

Family *Cladosporiaceae* Chalm. & R.G. Archibald

Genus *Cladosporium* Link

- *Cladosporium cladosporioides* (Fresen.) G.A. de Vries.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Kačániová et al., 2009; Nasser, 2004; Sinacori et al., 2013).
- *Cladosporium* sp. Link.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.

References: (Carvalho et al., 2020; Felsöciová et al., 2012; Grabowski & Klein, 2015; Kačániová et al., 2012; Kiš et al., 2018; Mašková et al., 2020).

Order *Dothideales* Lindau

Family *Saccotheciaceae* Bonord.

Genus *Aureobasidium* Viala & G. Boyer

- *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).
- *Aureobasidium* sp. Viala & G. Boyer.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Kačániová et al., 2012; Mašková et al., 2020).

Order *Incertae sedis*

Family *Seuratiaceae* Vuill. ex M.E. Barr

Genus *Atichia* Flot.

- *Atichia* sp. Flot..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Genus *Seuratia* Pat.

- *Seuratia* sp. Pat..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Order *Mytilinidiales* E. Boehm, C.L. Schoch & Spatafora

Family *Mytilinidiaceae* Kirschst.

Genus *Peyronelia* Cif. & Gonz. Frag.

- *Peyronelia* sp. Cif. & Gonz. Frag..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Order *Pleosporales* Luttr. ex M.E. Barr

Family *Pleosporaceae* Nitschke

Genus *Alternaria* Nees

- *Alternaria alternata* (Fr.) Keissl..
  Studied substrate: Nectar Honey; Honeydew.
  References: (Kačániová et al., 2009; Sinacori et al., 2013).
- *Alternaria multiformis* (E.G. Simmons) Woudenb. & Crous.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Alternaria* sp. Nees.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Grabowski & Klein, 2015; Kačániová et al., 2012; Kiš et al., 2018; Mašková et al., 2020).

Genus *Stemphylium* Wallr.

- *Stemphylium* sp. Wallr..

Studied substrate: Honey Blossom.
References: (Mašková et al., 2020).

Family *Didymellaceae* Gruyter, Aveskamp & Verkley

Genus *Epicoccum* Link

- *Epicoccum* sp. Link.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015; Kačániová et al., 2012).

Genus *Phoma* Sacc.

- *Phoma* sp. Sacc..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015; Kačániová et al., 2012).

Family *Torulaceae* Corda

Genus *Torula* Pers.

- *Torula mellis* Fabian & Quinet.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).
- *Torula* sp. Pers..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Class *Eurotiomycetes* O.E. Erikss. & Winka

Order *Ascosphaerales* Gäum. ex Benny & Kimbr.

Family *Ascosphaeraceae* L.S. Olive & Spiltoir

Genus *Ascosphaera* L.S. Olive & Spiltoir

- *Ascosphaera atra* Skou & K. Hackett.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Ascosphaera* sp. L.S. Olive & Spiltoir.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Genus *Bettsia* Skou

- *Bettsia alvei* (Betts) Skou ex Pitt, Lantz, Pettersson & Leong.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015; Rodríguez-Andrade et al., 2019).

Order *Chaetothyriales* M.E. Barr

Family *Cyphellophoraceae* Réblová & Unter.

Genus *Cyphellophora* G.A. de Vries

- *Cyphellophora jingdongensis* H. Yang & K.D. Hyde.
  Studied substrate: Nectar Honey.
  References: (Yang et al., 2018).

Order *Eurotiales* G.W. Martin ex Benny & Kimbr.

Family *Aspergillaceae* Link

Genus *Aspergillus* P. Micheli ex Haller.

- *Aspergillus discophorus* Samson, Zalar & Frisvad.
  Synonym: *Emericella discophora*.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).

- *Aspergillus nidulans* (Eidam) G. Winter.
  Synonym: *Emericella nidulans*.
  Studied substrate: Nectar Honey.
  References: (Nasser, 2004).
- *Aspergillus qinqixianii* Y. Horie, Abliz & R.Y. Li.
  Synonym: *Emericella qinqixianii*.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).
- *Aspergillus asperescens* Stolk.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Aspergillus candidus* Link.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Kačániová et al., 2009; Martins et al., 2003; Nasser, 2004).
- *Aspergillus clavatus* Desm..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Kačániová et al., 2012).
- *Aspergillus flavus* Link.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Kačániová et al., 2009; 2012; Martins et al., 2003; Nasser, 2004).
- *Aspergillus fumigatus* Fresen..
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Kačániová et al., 2009; 2012; Martins et al., 2003; Nasser, 2004).
- *Aspergillus montevidensis* Talice & J.A. Mackinnon.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Aspergillus niger* Tiegh..
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Kačániová et al., 2009; 2012; Martins et al., 2003; Nasser, 2004; Sinacori et al., 2013).
- *Aspergillus ochraceus* G. Wilh..
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Kačániová et al., 2019; 2012).
- *Aspergillus proliferans* G. Sm..
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).
- *Aspergillus pseudoglaucus* Blochwitz.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Aspergillus* sp. P. Micheli ex Haller.
  Synonym: *Emericella* sp. and *Eurotium* sp..

Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
References: (Carvalho et al., 2020; Felsöciová et al., 2012; Grabowski & Klein, 2015; Kačániová et al., 2012; Mašková et al., 2020).

- *Aspergillus spelunceus* Raper & Fennell.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).
- *Aspergillus terreus* Thom.
  Studied substrate: Nectar Honey.
  References: (Kačániová et al., 2009).
- *Aspergillus versicolor* (Vuill.) Tirab..
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Kačániová et al., 2019; 2012; Nasser, 2004).

Genus *Paecilomyces* Bainier

- *Paecilomyces* sp. Bainier.
  Studied substrate: Nectar Honey; Honey Blossom.
  References: (Kiš et al., 2018).

Genus *Penicillium* Link

- *Penicillium italicum* Wehmer.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).
- *Penicillium solitum* Westling.
  Synonym: *Penicillium crustosum*.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).
- *Penicillium apimei* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken.
  Studied substrate: Nectar Honey.
  References: (Barbosa et al., 2017).
- *Penicillium aurantiogriseum* Dierckx.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Kačániová et al., 2012).
- *Penicillium brevicompactum* Dierckx.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Grabowski & Klein, 2015; Kačániová et al., 2012).
- *Penicillium brocae* S.W. Peterson, Jeann. Pérez, F.E. Vega & Infante.
  Studied substrate: Nectar Honey.
  References: (Barbosa et al., 2017).
- *Penicillium camemberti* Thom.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Penicillium chrysogenum* Thom.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.

References: (Felsöciová et al., 2012; Grabowski & Klein, 2015; Kačániová et al., 2012).

- *Penicillium citrinum* Thom.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Barbosa et al., 2017; Kačániová et al., 2012; Rodríguez-Andrade et al., 2019).
- *Penicillium commune* Thom.
  Studied substrate: Nectar Honey.
  References: (Felsöciová et al., 2012).
- *Penicillium corylophilum* Dierckx.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Barbosa et al., 2017; Kačániová et al., 2012; Nasser, 2004; Rodríguez-Andrade et al., 2019; Sinacori et al., 2013).
- *Penicillium cravenianum* Visagie & K. Jacobs.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Penicillium decumbens* Thom.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).
- *Penicillium echinulatum* Biourge.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).
- *Penicillium expansum* Link.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Kačániová et al., 2012).
- *Penicillium griseofulvum* Dierckx.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Grabowski & Klein, 2015; Kačániová et al., 2012).
- *Penicillium* sp. Link.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Barbosa et al., 2017; Carvalho et al., 2020; Grabowski & Klein, 2015; Kačániová et al., 2019; 2012; Kiš et al., 2018; Martins et al., 2003; Mašková et al., 2020; Sinacori et al., 2013).
- *Penicillium meliponae* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken.
  Studied substrate: Nectar Honey.
  References: (Barbosa et al., 2017).
- *Penicillium mellis* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken.
  Studied substrate: Nectar Honey.

References: (Barbosa et al., 2017).

- *Penicillium polonicum* K.W. Zaleski.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Felsöciová et al., 2012; Sinacori et al., 2013).
- *Penicillium raistrickii* G. Sm..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Kačániová et al., 2012).
- *Penicillium sclerotiorum* J.F.H. Beyma.
  Studied substrate: Nectar Honey.
  References: (Barbosa et al., 2017).
- *Penicillium wotroi* Houbraken, López-Quint., Frisvad & Samson.
  Studied substrate: Nectar Honey.
  References: (Barbosa et al., 2017).

Genus *Talaromyces* C.R. Benj.

- *Talaromyces affinitatimellis* Rodr.-Andr., Stchigel & Cano.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Talaromyces basipetosporus* Stchigel, Cano & Rodr.-Andr..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Talaromyces brunneosporus* Rodr.-Andr., Cano & Stchigel.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Talaromyces funiculosus* (Thom) Samson, N. Yilmaz, Frisvad & Seifert.
  Synonym: *Penicillium funiculosum*.
  Studied substrate: Nectar Honey.
  References: (Nasser, 2004).
- *Talaromyces brasiliensis* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken.
  Studied substrate: Nectar Honey.
  References: (Barbosa et al., 2017).
- *Talaromyces scorteus* (Nakaz., Y. Takeda & Suematsu) S.W. Peterson & Jurjević.
  Studied substrate: Nectar Honey.
  References: (Barbosa et al., 2017).

Genus *Xerochrysium* Pitt

- *Xerochrysium xerophilum* (Pitt) Pitt.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).

Family *Monascaceae* J. Schröt.

Genus *Monascus* Tiegh.

- *Monascus mellicola* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken.
  Studied substrate: Nectar Honey.

References: (Barbosa et al., 2017).

- *Monascus pilosus* K. Satô ex D. Hawksw. & Pitt.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Monascus purpureus* Went.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Monascus ruber* Tiegh..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).

Order *Incertae sedis*

Family *Eremascaceae* Engl. & E. Gilg

Genus *Eremascus* Eidam

- *Eremascus albus* Eidam.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).

Order *Onygenales* Cif. ex Benny & Kimbr.

Family *Helicoarthrosporaceae* Stchigel, Rodr.-Andrade & Cano

Genus *Helicoarthrosporum* Stchigel, Cano & Rodríguez-Andrade

- *Helicoarthrosporum mellicola* Stchigel, Cano & Rodríguez-Andrade.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).

Family *Incertae sedis*

Genus *Strongyloarthrosporum* Rodr.-Andr., Cano & Stchigel

- *Strongyloarthrosporum catenulatum* Rodr.-Andr., Cano & Stchigel.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).

Class *Leotiomycetes* O.E. Erikss. & Winka

Order *Helotiales* Nannf.

Family *Incertae sedis*

Genus *Coniothecium* Corda

- *Coniothecium* sp. Corda.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Genus *Triposporium* Corda

- *Triposporium* sp. Corda.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Family *Myxotrichaceae* Locq. ex Currah

Genus *Oidiodendron* Robak

- *Oidiodendron mellicola* Rodr.-Andr., Cano & Stchigel.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).

Genus *Skoua* A.A. Wynns

- *Skoua asexualis* Rodr.-Andr., Cano & Stchigel.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Skoua fertilis* (Stoppel) A.A. Wynns.

Studied substrate: Honey Blossom; Honeydew.
References: (Rodríguez-Andrade et al., 2019).

Family *Sclerotiniaceae* Whetzel

Genus *Botrytis* Micheli ex Pers.

- *Botrytis* sp. P. Micheli ex Pers..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015; Kačániová et al., 2012).

Class *Saccharomycetes* G. Winter

Order *Incertae sedis*

Family *Incertae sedis*

Genus *Oosporidium* Stautz

- *Oosporidium* sp. Stautz.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Order *Saccharomycetales* Luerss.

Family *Incertae sedis*

Genus *Candida* Berkhout

- *Candida* sp. Berkhout.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).
- *Candida lundiana* Saks., M. Suzuki, Lumyong, Ohkuma & Chantaw.
  Studied substrate: Nectar Honey.
  References: (Saksinchai et al., 2012).
- *Candida parapsilosis* (Ashford) Langeron & Talice.
  Studied substrate: Nectar Honey.
  References: (Carvalho et al., 2010).
- *Candida suthepensis* Saks., M. Suzuki, Lumyong, Ohkuma & Chantaw..
  Studied substrate: Nectar Honey.
  References: (Saksinchai et al., 2012).

Genus *Starmerella* C.A. Rosa & Lachance

- *Starmerella magnoliae* (Lodder & Kreger-van Rij) C.A. Rosa & Lachance.
  Synonym: *Candida magnoliae*.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Carvalho et al., 2010; Rodríguez-Andrade et al., 2019).
- *Starmerella sorbosivorans* (S.A. James, C.J. Bond & I.N. Roberts) C.A. Rosa & Lachance.
  Synonym: *Candida sorbosivorans.*
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Carvalho et al., 2010; Rodríguez-Andrade et al., 2019).

Family *Lipomycetaceae* E.K. Novák & Zsolt

Genus *Lipomyces* Lodder & Kreger-van Rij

- *Lipomyces* sp. Lodder & Kreger-van Rij.

Studied substrate: Honey Blossom; Honeydew.
References: (Grabowski & Klein, 2015).

Family *Saccharomycetaceae* G.Winter

Genus *Debaryomyces* Klöcker

- *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).

Genus *Pichia* E.C. Hansen

- *Pichia* sp. E.C. Hansen.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).
- *Pichia membranifaciens* (E.C. Hansen) E.C. Hansen.
  Studied substrate: Nectar Honey.
  References: (Carvalho et al., 2010).

Genus *Saccharomyces* Meyen

- *Saccharomyces* sp. Meyen.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Grabowski & Klein, 2015; Martins et al., 2003).
- *Saccharomyces cerevisiae* (Desm.) Meyen.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Carvalho et al., 2010; Grabowski & Klein, 2015).

Genus *Schwanniomyces* Klöcker

- *Schwanniomyces* sp. Klöcker.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Genus *Zygosaccharomyces* B.T.P. Barker

- *Zygosaccharomyces favi* G. Péter, Čadež & Dlauchy.
  Studied substrate: Nectar Honey.
  References: (Čadež et al., 2015).
- *Zygosaccharomyces gambellarensis* Torriani, M. Lorenzini, Salvetti & Felis.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Zygosaccharomyces mellis* Fabian & Quinet.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Carvalho et al., 2010; Grabowski & Klein, 2015; Rodríguez-Andrade et al., 2019; Sinacori et al., 2013).
- *Zygosaccharomyces priorianus* Klöcker.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).
- *Zygosaccharomyces rouxii* (Boutroux) Yarrow.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.

References: (Carvalho et al., 2010; Grabowski & Klein, 2015).

- *Zygosaccharomyces siamensis* Saks., M. Suzuki, Chantaw., Ohkuma & Lumyong.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Rodríguez-Andrade et al., 2019; Saksinchai et al., 2012).
- *Zygosaccharomyces* sp. B.T.P. Barker.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Family *Trichomonascaceae* Kurtzman & Robnett

Genus *Blastobotrys* Klopotek

- *Blastobotrys meliponae* R.N. Barbosa, Boekhout, G.A. Silva, Souza-Motta & N. Oliveira.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Crous et al., 2016).

Family *Wickerhamomycetaceae* Kurtzman, Robnett & Bas.-Powers

Genus *Wickerhamomyces* Kurtzman, Robnett & Bas.-Powers

- *Wickerhamomyces* sp. Kurtzman, Robnett & Bas.-Powers.
  Synonym: *Hansenula* sp..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Class *Schizosaccharomycetes* O.E. Erikss. & Winka

Order *Schizosaccharomycetales* O.E. Erikss.

Family *Schizosaccharomycetaceae* Beij. ex Klöcker

Genus *Schizosaccharomyces* Lindner

- *Schizosaccharomyces* sp. Lindner.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).
- *Schizosaccharomyces octosporus* Beij..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Class *Sordariomycetes* O.E. Erikss. & Winka

Order *Hypocreales* Lindau

Family *Cordycipitaceae* Kreisel ex G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora

Genus *Engyodontium* de Hoog

- *Engyodontium* sp. de Hoog.
  Studied substrate: Nectar Honey.
  References: (Carvalho et al., 2020).

Family *Hypocreaceae* De Not.

Genus *Trichoderma* Pers.

- *Trichoderma hamatum* (Bonord.) Bainier.
  Studied substrate: Nectar Honey.
  References: (Nasser, 2004).
- *Trichoderma* sp. Pers..
  Studied substrate: Honey Blossom; Honeydew.

References: (Grabowski & Klein, 2015; Kačániová et al., 2012).

Family *Incertae sedis*

Genus *Acremonium* Link

- *Acremonium* sp. Link.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Grabowski & Klein, 2015; Kačániová et al., 2009).

Genus *Sarocladium* W. Gams & D. Hawksw.

- *Sarocladium strictum* (W. Gams) Summerb..
  Synonym: *Acremonium strictum*.
  Studied substrate: Nectar Honey.
  References: (Nasser, 2004).
- *Sarocladium* sp. W. Gams & D. Hawksw..
  Synonym: *Cephalosporium* sp..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Family *Nectriaceae* Tul. & C. Tul.

Genus *Fusarium* Link

- *Fusarium* sp. Link.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Felsöciová et al., 2012; Kačániová et al., 2009; 2012; Grabowski & Klein, 2015).
- *Fusarium oxysporum* Schltdl.
  Studied substrate: Nectar Honey.
  References: (Nasser, 2004).

Family *Stachybotryaceae* L. Lombard & Crous

Genus *Stachybotrys* Corda

- *Stachybotrys* sp. Corda.
  Studied substrate: Nectar Honey; Honey Blossom.
  References: (Kiš et al., 2018).

Order *Incertae sedis*

Family *Apiosporaceae* K.D. Hyde, J. Fröhl., Joanne E. Taylor & M.E. Barr

Genus *Arthrinium* Kunze

- *Arthrinium* sp. Kunze.
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Mašková et al., 2020; Sinacori et al., 2013).

Order *Sordariales* Chadef. ex D. Hawksw. & O.E. Erikss.

Family *Chaetomiaceae* G. Winter

Genus *Botryotrichum* Sacc. & Marchal

- *Botryotrichum atrogriseum* J.F.H. Beyma.
  Studied substrate: Nectar Honey.
  References: (Nasser, 2004).

Genus *Chaetomium* Kunze

- *Chaetomium globosum* Kunze.
  Studied substrate: Nectar Honey; Honeydew.

References: (Sinacori et al., 2013).

- *Chaetomium* sp. Kunze.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Genus *Trichocladium* Harz

- *Trichocladium griseum* (Traaen) X. Wei Wang & Houbraken.
- Synonym: *Humicola grisea*.
  Studied substrate: Nectar Honey.
  References: (Nasser, 2004).

Order *Xylariales* Nannf.

Family *Hypoxylaceae* DC.

Genus *Daldinia* Ces. & De Not.

- *Daldinia concentrica* (Bolton) Ces. & De Not..
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).

Phylum *Basidiomycota* R.T. Moore

Class *Microbotryomycetes* R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw.

Order *Sporidiobolales* Doweld

Family *Sporidiobolaceae* R.T. Moore

Genus *Rhodotorula* F.C. Harrison

- *Rhodotorula* sp. F.C. Harrison.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).
- *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison.
  Studied substrate: Nectar Honey.
  References: (Carvalho et al., 2010).

Class *Tremellomycetes* Doweld

Order *Filobasidiales* Jülich

Family *Filobasidiaceae* L.S. Olive

Genus *Naganishia* Goto

- *Naganishia uzbekistanensis* (Á. Fonseca, Scorzetti; Fell) Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout.
  Synonym: *Cryptococcus uzbekistanensis*.
  Studied substrate: Nectar Honey; Honeydew.
  References: (Sinacori et al., 2013).

Order *Tremellales* Fr.

Family *Cryptococcaceae* Kütz. ex Castell. & Chalm.

Genus *Cryptococcus* Vuill.

- *Cryptococcus neoformans* (San Felice) Vuill..
  Synonym: *Cryptococcus neoformans* var. *grubii*.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Order *Trichosporonales* Boekhout & Fell

Family *Trichosporonaceae* Nann.

Genus *Cutaneotrichosporon* Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout

- *Cutaneotrichosporon mucoides* (E. Guého; M.T. Sm.) Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout.

Studied substrate: Nectar Honey.
References: (Carvalho et al., 2010).

Genus *Vanrija* R.T. Moore

- *Vanrija humicola* (Dasz.) R.T. Moore.
  Studied substrate: Nectar Honey.
  References: (Carvalho et al., 2010).

Class *Ustilaginomycetes* Warm.

Order *Ustilaginales* Bek.

Family *Ustilaginaceae* Tul. & C. Tul.

Genus Not determined.

- *Ustilaginaceae* sp. Tul. & C. Tul..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Grabowski & Klein, 2015).

Class *Wallemiomycetes* Zalar, de Hoog & Schroers

Order *Wallemiales* Zalar, de Hoog & Schroers

Family *Wallemiaceae* R.T. Moore

Genus *Wallemia* Johan-Olsen

- *Wallemia hederae* S. Jančič, Zalar & Gunde Cimerman..
  Studied substrate: Nectar Honey.
  References: (Jančič et al., 2015b).
- *Wallemia mellicola* Jancic, Nguyen, Seifert & Gunde-Cimerman..
  Studied substrate: Nectar Honey.
  References: (Jančič et al., 2015b).

Phylum *Mucoromycota* Doweld

Class *Mucoromycetes* Doweld

Order *Mucorales* Dumort.

Family *Cunninghamellaceae* Naumov ex R.K. Benj.

Genus *Cunninghamella* Matr.

- *Cunninghamella elegans* Lendn..
  Synonym: *Cunninghamella bertholletiae*.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).

Family *Mucoraceae* Fr.

Genus *Mucor* P. Micheli ex L.

- *Mucor hiemalis* Wehmer..
  Studied substrate: Nectar Honey.
  References: (Kačániová et al., 2009).
- *Mucor* sp. P. Micheli ex L..
  Studied substrate: Honey Blossom; Honeydew; Nectar Honey.
  References: (Grabowski & Klein, 2015; Kačániová et al., 2012; Kiš et al., 2018; Martins et al., 2003; Mašková et al., 2020).
- *Mucor plumbeus* Bonord..
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Mucor racemosus* Bull..
  Studied substrate: Nectar Honey.

References: (Kačániová et al., 2009).

Genus *Rhizopus* Ehrenb.

- *Rhizopus arrhizus* A. Fisch..
  Synonym: *Rhizopus oryzae*.
  Studied substrate: Honey Blossom; Honeydew.
  References: (Rodríguez-Andrade et al., 2019).
- *Rhizopus* sp. Ehrenb..
  Studied substrate: Nectar Honey.
  References: (Felsöciová et al., 2012).
- *Rhizopus stolonifer* (Ehrenb.) Vuill..
  Studied substrate: Nectar Honey.
  References: (Kačániová et al., 2009).

Note: *Peronosporaceae* sp. de Bary and *Pythium* sp. Pringsh., have also been isolated, identified and noted as fungi, from Honey Blossom and Honeydew samples (Grabowski & Klein, 2015).

*****The checklist was based on a survey of scientific papers that identify fungal taxa isolated from honey, honey blossom and honeydew, during the time period of 2000 to 2022 (leaving out older studies (e.g., Ruiz-Argueso & Rodriguez-Navarro, 1975; Lochhead & Farrell, 1931; Snowdon & Cliver, 1996)). While additional data obtained based on spore microscopical identification is available (e.g. Pérez-Atanes et al., 2001; Diez et al., 2004; Dimou et al., 2006; Seijo et al., 2011; Olga et al., 2012; Magyar et al., 2005, 2016; Mura-Mészáros & Magyar, 2017) an option to limit the checklist to works focusing on fungal isolation and identification using morphological and/or molecular methods was employed (Martins et al., 2003; Nasser, 2004; Kacániová et al. 2009, 2012; Carvalho et al., 2010; Saksinchai et al., 2012a, b; Felšöciová et al., 2012/2013; Sinacori et al., 2014; Čadež et al. 2015; Grabowski & Klein 2015; Jančič et al., 2015a, b; Crous et al., 2016; Barbosa et al., 2017, 2018; Kiš et al., 2018, 2019; Yang et al., 2018; Rodríguez-Andrade et al., 2019; Carvalho et al., 2020; Mašková et al., 2020). Moreover, the checklist was annotated to contain currently accepted fungal names according to Index Fungorum (www.indexfungorum.org).

1.4.4 Portuguese honey mycobiome

Studies related to the diversity of fungi present in honey samples are scarce in Portugal. To date, only eighteen species (Table 1.1) have been found across three main studies (Carvalho et al., 2010; Magyar et al., 2005; Martins et al., 2003). Moreover, from these, solely the study conducted by Carvalho and colleagues (2010) has employed molecular markers. These results are meager, considering that, for instance, in a sole study in the neighbor country, Spain, thirty-two species, including eight new taxa, were recently identified or described (Rodríguez-Andrade et al., 2019).

Table 1.1 – Fungal species identified in Portuguese honey samples.

Genus	Species	Reference
	Aspergillus candidus	Martins et al., 2003
Aspergillus	*Aspergillus flavus*	Martins et al., 2003
	Aspergillus fumigatus	Martins et al., 2003

	Aspergillus niger	Martins et al., 2003
Penicillium	*Penicillium* spp.	Martins et al., 2003
Mucor	*Mucor* spp.	Martins et al., 2003
Metschnikowia	*Metschnikowia reukaufii*	Magyar et al., 2005
Candida	*Candida parapsilopsis*	Carvalho et al., 2010
Saccharomyces	*Saccharomyces* sp.	Martins et al., 2003
	Saccharomyces cerevisiae	Carvalho et al., 2010
Zygosaccharomyces	*Zygosaccharomyces mellis*	Carvalho et al., 2010
	Zygosaccharomyces rouxii	Carvalho et al., 2010
Starmerella	*Starmerella magnoliae*	Carvalho et al., 2010
Starmerella	*Starmerella sorbosivorans*	Carvalho et al., 2010
Rhodotorula	*Rhodotorula mucilaginosa*	Carvalho et al., 2010
Cutaneotrichosporon	*Cutaneotrichosporon mucoides*	Carvalho et al., 2010
Vanrija	*Vanrija humicola*	Carvalho et al., 2010
Pichia	*Pichia membranifaciens*	Carvalho et al., 2010

Understandably, this poses a serious gap on the country's honey mycological characterization, with implications for basic scientific knowledge, industrial applications, food spoilage and even environmental pathogen monitoring (Figure 1.3).

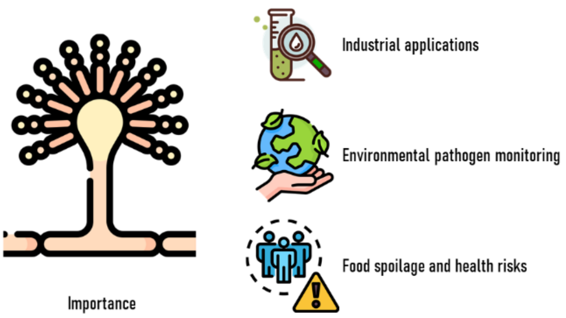


Figure 1.3 – Importance of honey fungi studies (constructed with icons from flaticon (https://www.flaticon.com/)).

1.5 Molecular mycology – general considerations

Modern analysis schemes in mycology are based on phenotypic and genotypic methods. Phenotypic methods for identifying fungi commonly involve plating on agar media followed by a macro and microscopic morphological examination. A limited selection of physiological and biochemical response patterns is also used as a phenotypic method. Disadvantages of microscopy are that it is time consuming, requires highly skilled technicians and makes identification beyond the genus level difficult. Fungi are a highly diverse group of organisms, with large numbers of species not yet described. The development of additional sequencing technology made it possible to study microbial communities in various settings (Mahmoud & Zaher, 2015; Hawksworth, 2001). Fungal taxonomy uses genotypic methods such as the Polymerase Chain Reaction (PCR) of amplified Deoxyribonucleic acid (DNA) using conserved sequences of mitochondrial and ribosomal genes and internal regions (Shapaval et al., 2013). DNA sequences are now an important source of information in almost all areas of mycology, including phylogeny, taxonomy and ecology (Nilsson et al., 2012; Stajich et al., 2009).

The last few years have seen the advent of DNA-based methods for identifying honey floral sources (Bell et al., 2016; Guertler et al., 2014; Hawkins et al., 2015; Valentini et al., 2010, Milla et al., 2022). DNA barcoding is the process of using DNA sequences from standardized genetic markers to identify eukaryotic species (Mahmoud & Zaher, 2015). Likewise, the microbial contents of honey can also be identified through DNA based methods (Anderson et al., 2013; Wirta et al., 2021). PCR amplification of specific sequences for the identification of microorganisms has become, nowadays, a common practice, mainly due to the relative easiness of execution and high reproducibility (Carvalho et al., 2010). Moreover, DNA barcoding is known to be a powerful tool for specimen identification that could potentially be applied for identifying various fungal groups, overcoming the diverse traditional criteria (and their limitations) used for detailing different fungal groups (Raja et al., 2017). Thus, fungal identification has undergone a deep revolutionary change, with the introduction of PCR methods.

Currently, a polyphasic approach comprising a combination of ecological, micromorphological, cultural and molecular analysis, is accepted as the most correct path leading to fungal identification (Raja et al., 2017). For fungi, the Internal Transcribed Spacer (ITS) region of the Ribosomal Deoxyribonucleic acid (rDNA), a ca. 450 - 650 base pair (bp.) region consisting of the two variable spacers ITS1 and ITS2 and the intercalary, highly conserved 5.8S gene (Begerow et al., 2010; Nilsson et al., 2012), is recognized as the official primary fungal barcode (Schoch et al., 2012) and has an important role in detecting the genetic differences between different fungal groups (Del-Prado et al., 2010; Mahmoud & Zaher, 2015). For recognizing species in some groups the Large Subunit (LSU) of the ribosomal genes is superior to the ITS (Mahmoud & Zaher, 2015; Schoch et al., 2011). This molecular barcode (ITS) was selected mainly due to the verified easiness of amplification, the presence of multiple tandemly repeated copies, the ability to distinguish distinct species and strongly defined barcode gap between inter- and intraspecific variation (Schoch et al., 2012; Xu, 2016). However, with the rising number of studies using this marker, some problems have also been identified and the ITS region is known to: (1) present some lack of sequence divergence or clear barcode gap in closely related clades; (2) present difficulties in performing correct alignments in divergent taxa (a result from their noncoding and nonfunctional nature); (3) present some degree of sequence heterogeneity among gene copies, resulting in poor Sanger chromatographs; (4) fail amplification of some specimens using "universal primers"; and (5) have intraspecific and intragenomic polymorphisms in some fungal groups (Schoch et al., 2012; Stielow et al., 2015; Xu, 2016). Thus, for strains unable to be identified with the ITS region, additional taxon-specific secondary and/or tertiary barcodes are needed for a correct DNA identification of fungi (e.g. Hofstetter et al., 2019; Lücking et al., 2020; Stielow et al., 2015). Despite these challenges, ITS combines the highest resolution for distinguishing closely related species with high PCR and sequencing success rates across a wide range of fungi (Schoch et al., 2012).

1.6 Next Generation Sequencing (NGS)

First-generation Sanger sequencing was constrained to regions of interest inside a given set of genes, with the amount and scope of DNA sequence that could be created in a sequencing run being technically limited (Kumar et al., 2019). When compared to Sanger sequencing, Next-generation sequencing (NGS), also known as High-throughput sequencing (HTS), refers to sequencing methods that have significantly boosted sequence output while decreasing cost and time requirements. This technology is used to determine the order of nucleotides in entire genomes, or targeted regions of DNA or RNA, involving the processing of a large number of DNA fragments in parallel, and performing millions to billions of individual sequencing reactions simultaneously (Ambardar et al., 2016; Kumar et al., 2019). This methodology, compared to others, shows a higher sensitivity to detect low-frequency variants, a faster turnaround time for high sample volumes, comprehensive genomic coverage, and a higher capacity with sample multiplexing (Schuster, 2008; Shendure & Ji, 2008).

Briefly, in NGS sequencing, the DNA is first broken down into manageable segments that, when assembled, provide a piece of nucleotide sequence, which can then be read. After a sequencing run, raw sequencing reads are reassembled into the proper order, either via *de novo* assembly or by matching to a known reference sequence. The workflow generally consists of three steps: sample preparation, nucleic acid sequencing and data interpretation (Figure 1.4), notwithstanding the enormous variations in NGS methodologies (Kumar et al., 2019). NGS can be used to sequence entire genomes or be constrained to specific areas of interest (Behjati & Tarpey, 2013). Currently, HTS has been widely used for functional microorganisms as well as universal gene analysis (Hou et al., 2018; Pan et al., 2018; Sultana et al., 2019), largely because it offers a large volume of information, is fast and easy to use and is currently not overly expensive (Wang & Yao, 2021).

Application of NGS to honey samples is a rapidly growing procedure, since it allows for multiple critical scientific, ecological and sanitary questions to be solved. Their main applications include (but are not limited to): (1) entomological authentication; (2) honey floral determination; (3) honey geographical discrimination; (4) exploration of bee foraging interactions and competition; (5) pest monitoring and detection; and (6) microbiome analysis and environmental impact evaluation.

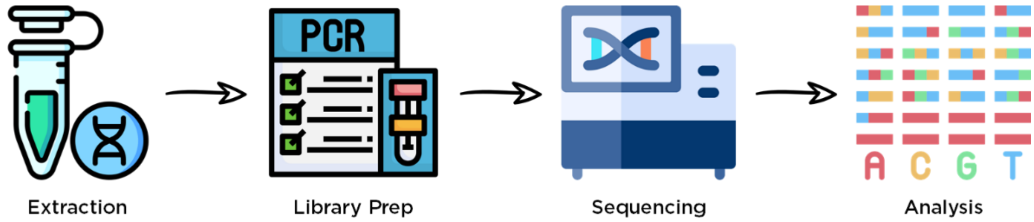


Figure 1.4 – Next-generation sequencing overview (constructed with icons from flaticon (https://www.flaticon.com/)).

1.7 Thesis aim

Although honey should be a substratum amenable for the growth of fungi allowing the development of highly specific fungi, particularly xerotolerant and xerophilic, few studies have intentionally targeted these fungi. Therefore, the first objective of this study is to provide a preliminary evaluation of the microbiological contamination of Portuguese monofloral honey and characterize their fungal diversity (through both classic cultivation and NGS methods). The second objective is to determine whether xerophilic fungi are present in a group of raw honey samples, particularly from local and commercial producers, from various botanical and geographic origins, using culture and non-culture dependent methods. It is also intended to characterize the morphology, physiology and phylogeny of new isolates considered of taxonomic interest, and thus contribute towards the microbiological knowledge of Portuguese monofloral honey. Finally, this work also focuses on the impact of honey mycobiota along the food chain and evaluates the pathogenic potential of the microorganisms found in honey.

2 MATERIALS AND METHODS

2.1 Sample description and collection

A total of 3 monofloral raw honey samples were collected from different locations in Portugal (Figure .1), either directly from a beekeeper (local producer), or from a trade network (commercial producer) located in the south and center of Portugal, respectively. In addition, the source of the nectar also varied between samples, with two different sources studied, namely rosemary (*Lavandula* spp.) and eucalyptus (*Eucalyptus* spp.).

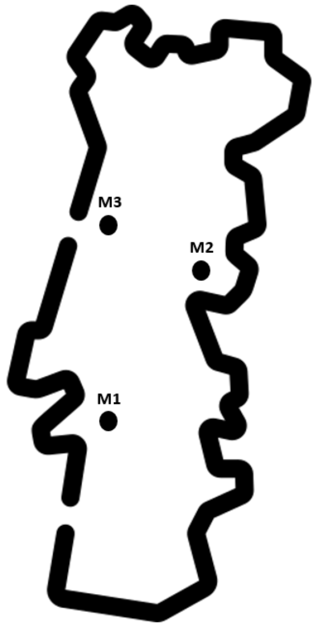


Figure 2.1 – Locations where the samples were collected. Region of Setúbal (M1), Region of Beira Baixa (M2) and Region of Beira Litoral (M3) (constructed with icons from flaticon (https://www.flaticon.com/)).

The eucalyptus sample (M3), originating from the nectar of eucalyptus flowers from Beira Litoral, had a light brown tone. The samples of rosemary, originating from the predominantly rosemary flowering, were collected from the inner region of Beira Baixa (M2) and the region of Setúbal (M1), and had a light amber tone and a high concentration of fructose (Figure .2).

Figure 2.2 – Monofloral honey samples collected from a local producer (M1) and commercial producer (M2 and M3).

None of the samples were heated or pasteurized by the producers and showed no signs of fermentation or crystallization. In addition, all the samples were processed in 2022 and presented no apparent signs of alteration. All packaged samples were transported and stored at room temperature (20°C). The De Moraes & Teixeira, (1998) method was used to determine honey pH levels. An analytical scale was employed to weigh 10 g of honey in a 100 mL beaker, with the sample further diluted with 75 mL of distilled water. Next, the pH was determined by a *3510 Standard Digital pH Meter* (Jenway, UK), calibrated with neutral (pH 7,0) and acidic (pH 4,0) buffers, for each independent honey sample. Additional details regarding the samples used in this study are summarized in Table 2.1.

Table 2.1 – List and details of examined honey samples.

Honey reference number	Locality	Region	Honey type	Source of the nectar	Sample (g)	Pollinator	pH
M1	Region of Setúbal	South	Monofloral	Rosemary (*Lavandula* spp.)	500	*Apis melífera* (sp. iberica)	3,90 ± 0,01
M2	Region of Beira Baixa	Center	Monofloral	Rosemary (*Lavandula* spp.)	280	*Apis melífera* (sp. iberica)	3,94 ± 0,01
M3	Region of Beira Litoral	Center	Monofloral	Eucalyptus (*Eucalyptus* spp.)	280	*Apis melífera* (sp. iberica)	4,40 ± 0,01

2.2 Isolation of fungi from the stored honey

For each honey sample, a 1 mL solution composed of honey and ultrapure water was prepared by dissolving 100 mg of honey in 900 μL of MilliQ water (previously sterilized). The homogenization of each of the solutions was performed by hand for 2 minutes. The solutions prepared for each sample were aseptically plated in triplicate on Petri dishes with a diameter of 90 mm containing the employed media, namely Potato Dextrose Agar (PDA) (VWR Chemicals, EUA) and Dichloran Glycerol Agar (DG18) (Sigma-Aldrich, USA). In each Petri dish, 200 μL of the previously prepared honey solution (0,2 mL/plate) was inoculated and distributed over 12-15 equally spaced points (Figure .3). The plates were then incubated aerobically in the dark at 25 ± 1°C for 2 months, and observed at 24-h intervals for the quantification and identification of new colonies.

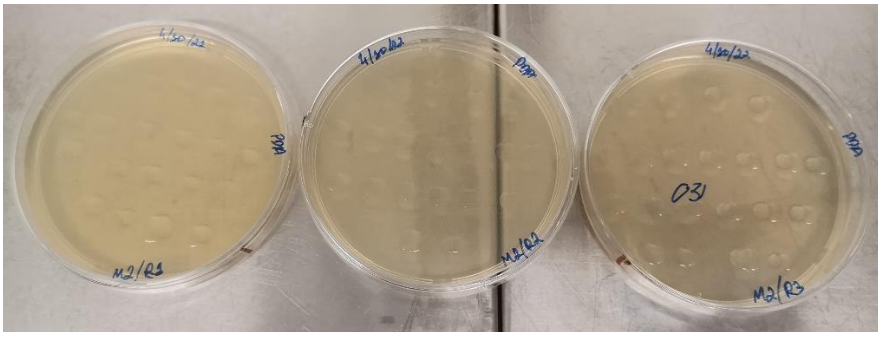


Figure 2.3 – Representation of the inoculation technique in PDA medium.

2.3 Fungal colony isolation and initial identification

The fungi were initially evaluated by their culture and micromorphological features (Samson et al., 2010). Such features included the fungal structures representative of all morphological varieties (morphotypes) of the organisms of interest, namely the colonies appearance (color, texture, diffusible pigments, exudates, growth zones, aerial and submerged hyphae, growth rate and topography) once they reached a diameter of approximately 2 mm. Next, they were isolated into 60 mm Petri dishes containing the original culture medium and incubated at 25 ± 1°C in the dark for 5-7 days to obtain pure cultures. At the same time, each isolated colony was observed under 40X and 100X magnifications in a *Leica DME* (Leica, Germany) light microscope for morphological characterization and identification. Finally, the most abundant, or interesting isolated fungal species were grouped based on appearance and prepared for total DNA extraction, molecular sequencing and analysis in an attempt to identify each species.

2.4 DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

DNA from pure fungal cultures was obtained using the Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, USA) with some modifications to the standard protocol. A small portion of the colonies was scraped from the agar surface using a sterile scalpel, submerged in 10 μL of extraction solution and incubated in an ABI GeneAmp 9700

PCR System (Applied Biosystems, USA), with the following protocol: 65°C for 10 min, followed by 95°C for 15 min. After the incubation, reactions were stopped by adding 10 µL of dilution solution and the resulting mixture was vortexed for 2 min. The extracted DNA was subjected to PCR amplification of the ITS-rDNA region, by using the universal fungal primer pair ITS1-F/ITS4 (Table 2.2) (Gardes & Bruns, 1993; White et al., 1990). Overall, the PCR reactions were conducted with a final volume of 25 µL, consisting of 12,5 µL of NZYTaq Green Master Mix (NZYTech™, Portugal), 1 µL of each primer (10 mM), 9,5 µL of ultra-pure water and 1 µL of template DNA in an ABI GeneAmp 9700 PCR System (Applied Biosystems, USA). PCR conditions were as follows: initial denaturation at 96°C for 45 s, followed by 40 cycles of denaturation at 96°C for 45 s, annealing at 54°C for 90 s, and extension at 72°C for 90 s, with a final extension at 72°C for 2 min. After PCR, visual confirmation of the overall amplification of the ITS region was performed with a simple agarose gel electrophoresis (Figure .4) using 1,2% (w/v) agarose in 1× TBE buffer stained with GreenSafe Premium (NZYTech™, Portugal) and visualized in a Molecular Imager Bio-Rad Gel Doc XR™ (Bio-Rad, Hercules, California, USA).

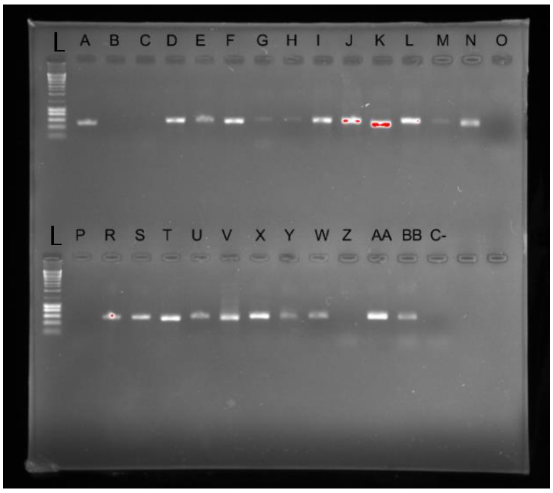


Figure 2.4 – PCR amplification of the ITS region performed for the isolates in study.

Considering that, in some cases, the amplification of the ITS-rDNA region failed, or was not useful for identification, the 28S (LSU) and *benA* (β-tubulin) genes were amplified instead. For these cases, the primer pairs LSU1fd/LR5 and BT2a/BT2b were respectively used (Table .2) (Crous et al., 2009; Vilgalys & Hester, 1990), alongside a similar PCR program as the one described above, though with slight modifications (see Table .2). For PCR products purification, the *EXO/SAP Go PCR Purification Kit* (GRISP, Portugal) was used with some adjustments. Amplicons were then sequenced using an *ABI 3730xl DNA Analyzer* system (96 capillary instruments) at *Eurofins Genomics*, Germany (https://eurofinsgenomics.eu/).

Table 2.2 – Primers used in the current study.

Locus	Primer name	Primer sequence (5'-3')	Annealing temperature used (°C)	Reference
β-tubulin	BT2a (F)	GGTAACCAAATCGGTGCT GCTTTC	52	(Glass & Donaldson, 1995)
	BT2b (R)	ACCCTCAGTGTAGTGACC CTTGGC		
ITS	ITS1F (F)	CTTGGTCATTTAGAGGAA GTAA	56	(Gardes & Bruns, 1993; White et al., 1990)
	ITS4 (R)	TCCTCCGCTTATTGATATG C		
LSU	LSU1Fd (F)	GRATCAGGTAGGRATACC CG	52	(Crous et al., 2009; Vilgalys & Hester, 1990)
	LR5 (R)	TCCTGAGGGAAACTTCG		

2.5 Molecular analysis and fungal identification

Obtained DNA sequences were processed with the Geneious® R11.0.02 software and molecular similarity comparisons were performed using the National Center of Biotechnology Information nucleotide database (NCBI's) Basic Local Alignment Search Tool (BLAST), with the option Standard nucleotide BLASTn of BLAST 2,6 (Altschul et al., 1997). Moreover, a second round of molecular analysis was also conducted with the online MycoBank database (Crous et al., 2004; Robert et al., 2005) with the Molecular ID Pairwise Alignment tool (https://www.mycobank.org/page/Pairwise_alignment) with the default parameters and all databases selected. Taking into account the results obtained a complementary analysis of macro and microscopic fungal taxonomic traits was conducted, to ensure that a proper species identification was achieved. Nonetheless, species with low asexual/sexual characters differentiation, thus limiting morphological confirmation or potentially new (undescribed), were marked as sp..

2.6 Morphological identification - Slide culture technique

One of the cornerstone techniques for the morphological identification of fungi in diagnostic mycology continues to be the conventional slide culture technique for the close observation of undisturbed microscopic features of filamentous fungi. This procedure follows the steps described by Riddell (1950), allowing a more detailed study of the isolates *in situ* with as little disturbance as possible. It is a quick way of producing fungal colonies for examination and identification, with the benefit that no portion of the fungus needs to be removed from the culture plate and transferred to the slide, decreasing the likelihood that the traits essential for identification will be lost (e.g., spore-bearing structures). Conidial fungi can be seen up close using this technique, which also clearly defines the essential morphological details necessary for filamentous fungi identification (Prakash & Bhargava, 2016).

For the staining and microscopic identification of fungi, a simple histological staining method based on Lactophenol cotton blue (LPCB) was used. The Lactophenol cotton blue solution acts as a mounting solution, as well as a staining agent. The preparation has three components: phenol, which kills any live organisms; lactic acid, which preserves fungal structures; and cotton blue, which stains the chitin of the fungal cell walls and other fungal structures. With this process, the morphology of fungi can be visually inspected, providing a tool for potential classification and identification (Leck, 1999; Mokobi, 2022).

For the isolates of interest, a sterile rod was placed on sterile filter paper and aseptically introduced into a Petri dish. A sterilized slide was placed on 2 supports after the filter paper had been thoroughly moistened with sterile water. A 5 mm square block of PDA media was transferred to the center of the slide and inoculated in all four corners of the agar square with the mycelium from the selected fungus. Next, a sterile cover glass was placed aseptically on top of the block of PDA, and the inoculum was incubated at 25 ± 1°C, for 7 days, to promote hyphal development and the formation of spores (Figure .5). Finally, a drop of LPCB stain was placed on a clean microscope slide, and the cover glass was removed from the slide culture and placed on the drop of LPCB for posterior examination of the slide under a *Nikon Eclipse Ci-L* (Nikon, Minato, Japan) light microscope.

Figure 2.5 – Slide culture preparation for morphological identification.

2.7 DNA extraction and High-Throughput Sequencing (HTS)

DNA was extracted from 3 honey samples using a sample pre-treatment procedure (Soares et al., 2015; Waiblinger et al., 2012) and *DNeasy PowerLyzer PowerSoil kit* (Qiagen, Hilden, Germany), according to the manufacturer's instructions with minor modifications. Samples were prepared for Illumina Sequencing by the amplification of the *Internal Transcribed Spacer 2* region of the mycobiota.

The DNA was amplified for the hypervariable regions with specific primers, and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. A first set of PCR reactions was performed for each sample using the *KAPA HiFi HotStart PCR kit* according to the manufacturer's suggestions, 0,3 µM of each PCR primer: a pool of 6 forward primers plus 1 reverse primer (Table .3) (Tedersoo et al., 2014) and 10 µL of template DNA for a total volume of 25 µL. The PCR conditions involved a 3 min denaturation at 95°C, followed by 40 cycles at 98°C for 20 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. The second set of PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures.

Table 2.3 – HTS primers used in the current study.

Locus	Primer name	Primer sequence (5'-3')	Reference
ITS	ITS3NGS1 (F)	CATCGATGAAGAACGCAG	(Tedersoo et al., 2014)
	ITS3NGS2 (F)	CAACGATGAAGAACGCAG	
	ITS3NGS3 (F)	CACCGATGAAGAACGCAG	
	ITS3NGS4 (F)	CATCGATGAAGAACGTAG	
	ITS3NGS5 (F)	CATCGATGAAGAACGTGG	
	ITS3NGS10 (F)	CATCGATGAAGAACGCTG	
	ITS4NGS001 (R)	TCCTSCGCTTATTGATATGC	

PCR products were then one-step purified and normalized using *SequalPrep* 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau, Douglas, & Langille, 2017), pooled and pair-end sequenced in the *Illumina MiSeq®* sequencer with the *MiSeq Reagent kit v3*, according to the manufacturer's instructions (Illumina, San Diego, CA, USA) at *Genoinseq* (Cantanhede, Portugal).

2.8 Bioinformatic data analysis

Sequence data was initially processed at *Genoinseq* (Cantanhede, Portugal). Raw reads were extracted from the *Illumina MiSeq® System* in *fastq* format and quality-filtered with PRINSEQ *0.20.4* (Schmieder & Edwards, 2011) to remove sequencing adapters, trim bases with an average quality lower than 25% in a window of 5 bases, and eliminate reads with less than 100 bases for the ITS2 region and 150 bases for the 16S rRNA gene. The forward and reverse reads were merged by overlapping paired-end reads with *AdapterRemoval 2.1.5* (Schubert et al., 2016) using default parameters. The *ITSx* software (Bengtsson-Palme et al., 2013) was then applied to extract the ITS2 sub-region, while chimeric sequences were detected and removed from the dataset and the remaining reads clustered into Operational Taxonomic Units (OTUs) at 97% similarity employing the USEARCH/UPARSE approach (Edgar, 2013). Fungal OTUs taxonomic classification was achieved through BLAST analysis against the *UNITE database v.8* (Nilsson et al., 2019). Non-target OTUs, and with less than a minimum of three reads, were discharged from the dataset.

Abundance plots and statistical analysis were obtained in *Explore Metabar 2.0.1* (Rifa & Theli 2022), while shared OTUs Venn diagrams were obtained with the online *Van de Peer Lab tool* for that purpose (http://bioinformatics.psb.ugent.be/beg/tools/venndiagrams).

3 RESULTS AND DISCUSSION

3.1 General culture dependent methods data analysis

The applied culture dependent methods allowed for each of the colonies initially detected to be registered and isolated for posterior identification, based on their morphological and molecular characteristics (Figure 3.). A total of 110 colonies were accounted in the two-culture media used in this study (PDA and DG18). Overall, PDA showed a higher number of colonies than DG18, registering 60 (54,55%) and 50 records (45,45%), respectively.

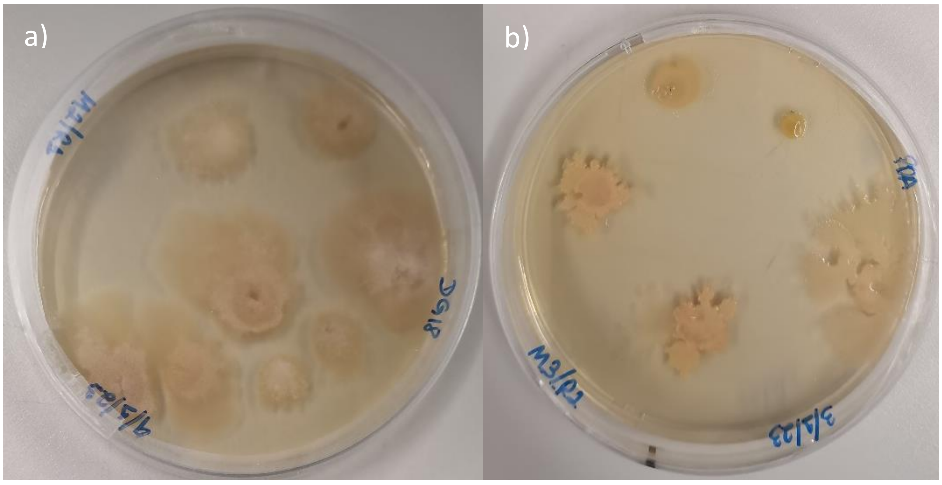


Figure 3.1 – Colonies developing after inoculations in (a) DG18 and (b) PDA media.

According to honey sample, M1 developed the most colonies, with a total of 50 (45,45%) records, followed by M2 with 42 (38,18%) and M3 with 18 (16,36%). In terms of culture media (Figure 3.2 – Number of colonies per honey sample depending on the culture media considered (constructed with elements from canva (https://www.canva.com/)).

), the M1 sample exhibited 34 colonies in PDA (and solely 16 in DG18), whereas the M2 sample exhibited 21 colonies in both media. On the other hand, the M3 sample presented a total of 13 records in DG18, and solely 5 in PDA.

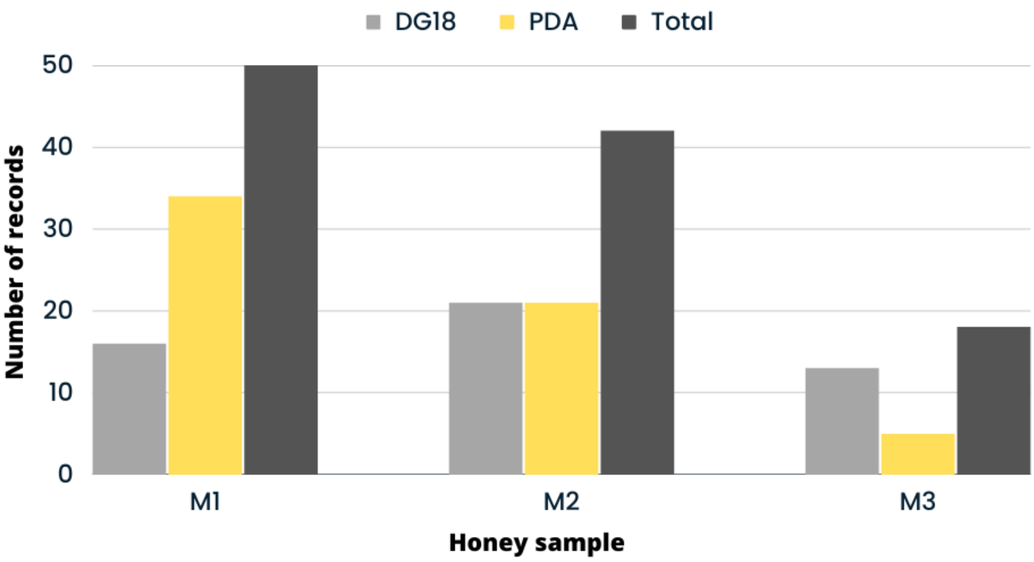
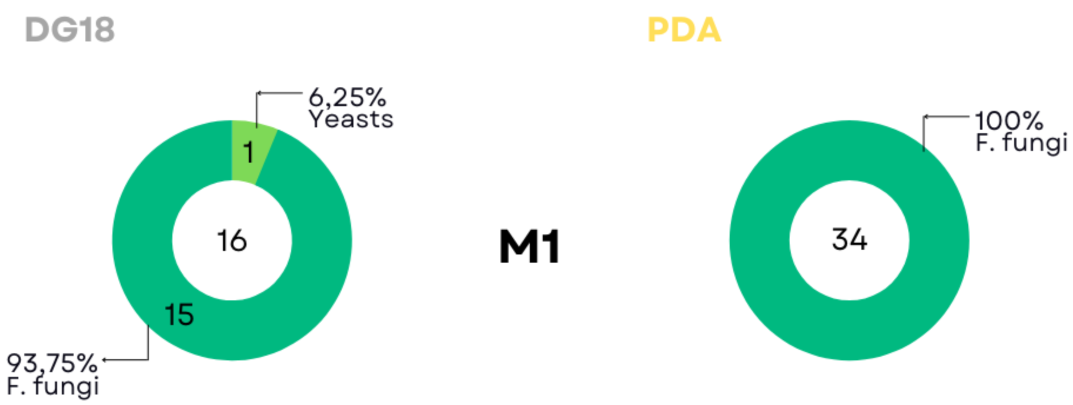


Figure 3.2 – Number of colonies per honey sample depending on the culture media considered (constructed with elements from canva (https://www.canva.com/)).

3.2 Fungal isolates data analysis

Figure 3.3 – Prevalence of filamentous fungi and yeasts in the honey samples regarding the culture media considered (constructed with elements from canva (https://www.canva.com/)).

.3 summarizes the distribution of fungal isolates by honey sample and prevalence in each of the corresponding culture media. The analysis of the considered honeys revealed that they all contained both fungi and yeasts. The data for the M1 sample revealed 34 filamentous fungi isolates (68,0%) and no evidence of yeasts regarding the inoculations in PDA media. Additionally, the DG18 media showed a prevalence of filamentous fungi with 15 isolates (93,75%) against only 1 yeast (6,25%). The distribution for the M2 sample is very similar to the M1 sample, differing mostly in the presence of yeasts on PDA (2 records - 9,52%). The M3 sample showed a somewhat similar occurrence of filamentous fungi and yeasts, with the DG18 medium showing a higher number of yeasts with 7 records (53,85%).



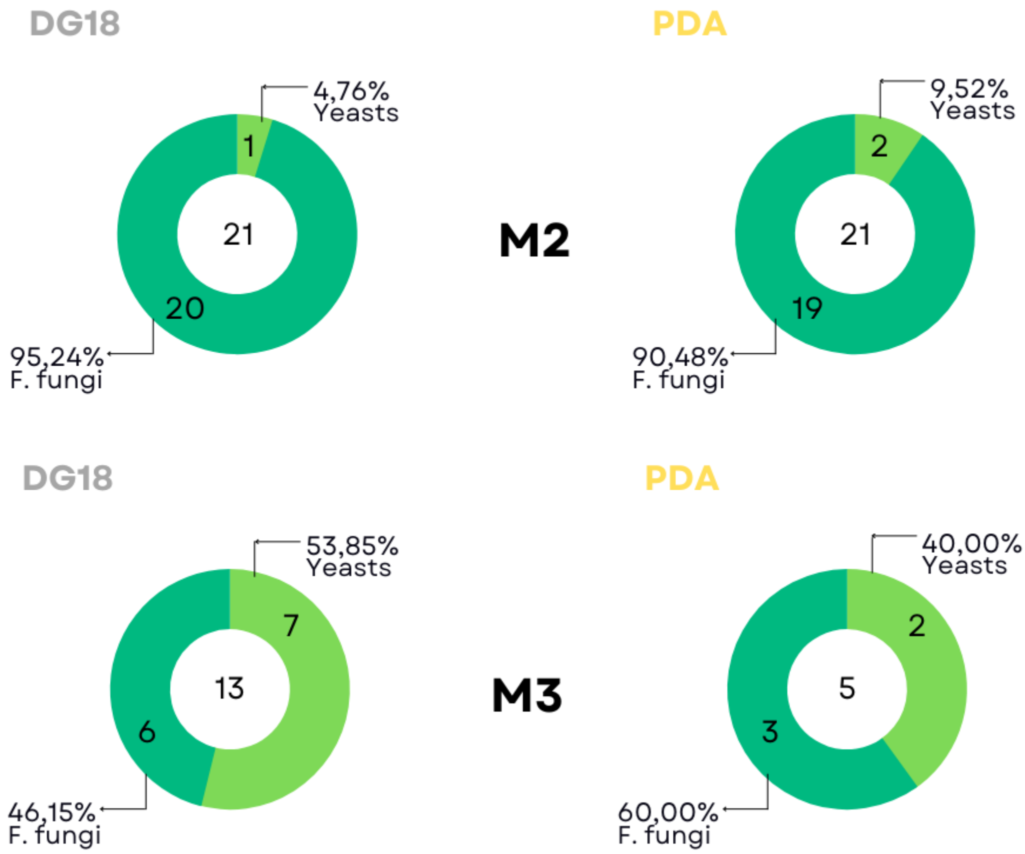


Figure 3.3 – Prevalence of filamentous fungi and yeasts in the honey samples regarding the culture media considered (constructed with elements from canva (https://www.canva.com/)).

Given the existence of contaminations in the 110 colonies originally obtained, only a total of 86 isolates were initially classified according to their morphological characteristics, 79 as filamentous fungi (91,86%) and 7 as yeasts (8,14%).

3.3 Fungal diversity according to botanical sources data analysis

The fungal diversity was also studied taking into account the botanical sources of each sample. The results (Figure 3.4 – Prevalence of isolates regarding the botanical sources and number of isolates found in the samples studied (constructed with elements from canva (https://www.canva.com/)).

.4) showed that the honeys derived from *Lavandula* spp. (M1 and M2), when compared with *Eucalyptus* spp. (M3), exhibited a higher number of fungal colonies. The sample M1 (*Lavandula* spp.) was the more diverse among the honeys, with a total of 45 isolates divided by 8 different genera. In comparison, samples M2 and M3 presented a lower number of isolates, with 29 (divided into 8 genera) and 12 (divided into 4 genera), respectively.

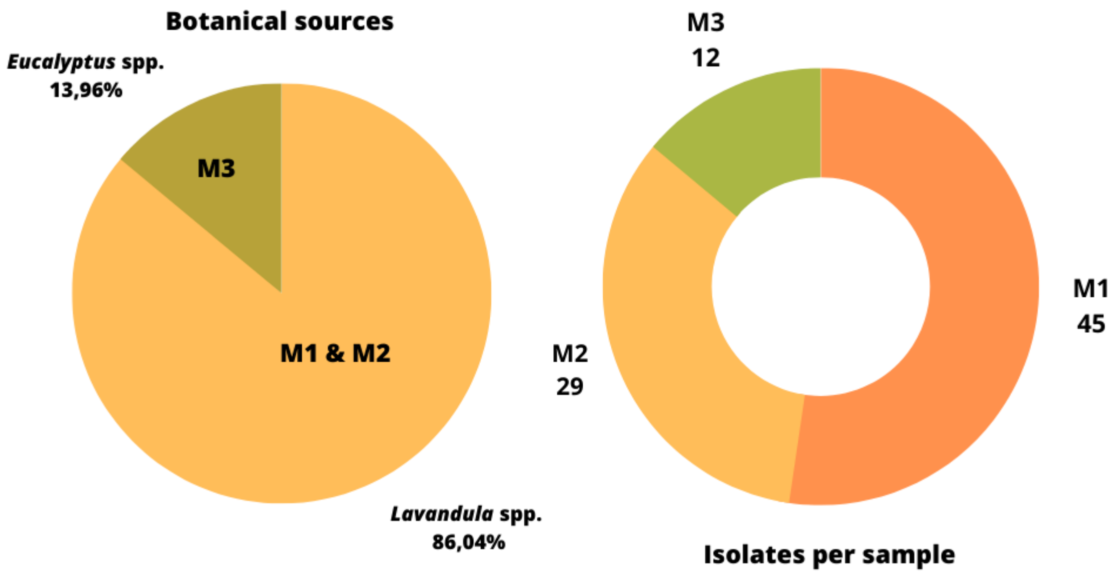


Figure 3.4 – Prevalence of isolates regarding the botanical sources and number of isolates found in the samples studied (constructed with elements from canva (https://www.canva.com/)).

3.4 Fungal molecular analysis

From a molecular point of view, the 86 isolated colonies were identified by sequencing the ITS rRNA gene, 28S gene (LSU) or even *benA* (β-tubulin) gene depending on the taxonomic group studied. This analysis allowed to recognize 18 fungal species belonging to 11 genera. Overall, the molecular analysis coupled with morphological confirmation, allowed the identification of the following species: *Alternaria alstroemeriae*, *Alternaria alternata*, *Aspergillus brunneus*, *Aspergillus* sp. (possible new species in sugenus *Aspergillus*; section *Aspergillus*: series *Aspergillus* and pending further studies (Houbraken et al., 2020)), *Bettsia alvei*, *Cladosporium cladosporioides*, *Epicoccum layuense*, *Meyerozyma caribbica*, *Penicillium glabrum*, *Penicillium janczewskii*, *Penicillium pancosmium*, *Penicillium raphiae*, *Pseudopithomyces rosae*, *Skoua fertilis*, *Talaromyces speluncarum*, *Trichoderma hamatum*, *Trichoderma harzianum* and *Trichoderma scalesiae*. Table 3. and Figure 3.5 summarize the fungal strains identified based on phenotypic and molecular data.

Table 3.1 – Fungal taxa identified with the closest match nucleotide sequence accession number, number of isolates and source of isolation.

Species	GenBank nucleotide sequence accession number (closest match)			Source of isolation	# isolates
	ITS (% identity[a])	LSU (% identity[a])	*benA* (% identity[a])		
Alternaria alstroemeriae	NR_163686.1 (100)	---	---	M1	5
Alternaria alternata	MH863109.1 (100)	MH866262.1 (100)	---	M1	2

Aspergillus brunneus	NR_163667.1 (99,79)	---	---	M2	1
***Aspergillus* sp.**	NR_135339.1 (99,46)	---	EF651891.1 (99,41)	M2	2
Bettsia alvei	MH862269.1 (100)	MH873951.1 (99,19)	---	M2	8
Cladosporium cladosporioides	NR_119839.1 (100)	---	---	M1	5
Epicoccum layuense	NR_158265.1 (100)	---	---	M1	4
Meyerozyma caribbica	NR_149348.1 (100)	---	---	M2; M3	7
Penicillium glabrum	NR_163530.1 (100)	---	---	M1; M2	6
Penicillium janczewskii	NR_111132.1 (100)	---	---	M3	3
Penicillium pancosmium	NR_121506.1 (100)	---	---	M1; M2; M3	16
Penicillium raphiae	---	NG_067463.1 (100)	---	M2	1
Pseudopithomyces rosae	NR_157539.1 (100)	---	---	M1	8
Skoua fertilis	---	NG_059988.1 (100)	---	M2	3
Talaromyces speluncarum	NR_172273.1 (100)	---	---	M2	7
Trichoderma hamatum	NR_134371.1 (99,78)	---	---	M1	4
Trichoderma harzianum	MH855340.1 (100)	---	---	M1	1
Trichoderma scalesiae	NR_144876.1 (100)	NG_069886.1 (100)	---	M1; M3	3

[a]According to BlastN search in the NCBI database and the Mycobank Molecular ID Pairwise Alignment tool.

Alternaria is a common fungal genus that causes pre- and postharvest damage to agricultural products, being also found on nectarivorous insects and often associated with the intestines of honeybees (Kiš et al., 2018). Many *Alternaria* species have the ability to generate secondary metabolites, namely phytotoxins (substances poisonous or toxic to plants) that, when present, can ruin a wide range of foods (Lopez & Cabral, 1999; Patriarca et al., 2014). Practically any form of substrate can support these saprophytic species. The presence of species of this genus has already been described in different honey subtracts (e.g., *A. alternata* in nectar honey and honeydew *A. multiformis* in honey blossom and honeydew (Rodríguez-Andrade et al., 2019)). The isolation of *A. alternata* (M1, Figure 3.5 c-d) is in accordance with the results present in the literature. Nonetheless, the isolation of *A. alstroemeriae* (M1, Figure 3.5 a-b) is a new record regarding the presence of this organism in honey subtracts.

The majority of *Aspergillus* (Figure 3.5 g-h) species are saprophytic fungus frequently found in soils and on various organic and inorganic substrates. A number of species from this genus are facultative parasites and have been also reported as agents of stonebrood (a low virulence disease in honey bee colonies caused by the fungi *Aspergillus flavus* and *Aspergillus fumigatus*) (Cha et al., 2021; Foley et al., 2014; Shoreit & Bagy, 1995). *Aspergillus* and *Penicillium* contain some of the most xerotolerant fungal species, and they collect internal glycerol as a suitable solute to counteract the environment's low a_W (Coleine et al., 2022; Fontana, 2020). They are, also known as main producers of mycotoxins (e.g., ochratoxin), thus being important food-spoilage microorganisms, while also posing some human health threats (Kozakiewicz, 1989; Pitt & Hocking, 1997). The presence of species of this genus has already been described in different honey subtracts (e.g., *A. flavus* in honey blossom, nectar honey and honeydew and *A. pseudoglaucus* in honey blossom and honeydew), with this genus also being considered as an environmental contaminant for honey (Kacániová et al., 2009). The isolation of *A. brunneus* (M2, Figure 3.5 e-f), during the course of this work, is thus, in accordance with the available literature, having been reported in honey samples by Silva et al. (2017).

Bettsia is a fungi genus within the *Ascosphaeraceae* family. *Bettsia* produce predominantly aleurioconidia (a thallic conidium formed by the breakdown of the supporting cell). This is a monotypic genus, containing the single species *B. alvei* (M2, Figure 3.5 i-j), that, in terms of ecology, has been isolated from a range of low water activity substrates, including honeycombs (Pitt & Hocking, 2009). According to the literature, this specie was found in honey blossom and honeydew subtracts (Grabowski & Klein, 2015; Rodríguez-Andrade et al., 2019), however this study highlights its presence in nectar honey as a new record.

Cladosporium species have a global range, being regularly found associated to various plants, and frequently isolated from soil, food and organic substrates (Bensch et al., 2012). Their presence is also found on nectarivorous insects, alongside *Alternaria* species, being also often associated with the intestines of honeybees (Kiš et al., 2018).The presence of species of this genus has already been described in various honey subtracts, with known previous reports of the isolation of *C. cladosporioides* in nectar honeys and honeydew (Kačániová et al., 2009; Nasser, 2004; Sinacori et al., 2013). The isolation of *C. cladosporioides* (M1, Figure 3.5 k-l) during the course of this work is, thus, in accordance with the available literature.

Epicoccum species are common endophytic and ubiquitous ascomycetes belonging to the *Didymellaceae* family (De Gruyter et al., 2009). They can be found in air, soil, associated with various plant parts (flowers, twigs, leaves and bark) and water (Arenal et al., 1999; Chen et al., 2017; Taguiam et al., 2021). Aside from the diversity of habitats, *Epicoccum* gathers important fungal species due to their ability to produce mycotoxins and present antifungal activity (Braga et al., 2018). The fungal colonies of *E. layuense* (M1, Figure 3.5 m-n) represent a new record regarding nectar honey. However, *Epicoccum* sp. has already been found in honey subtracts (e. g., honey blossom and honeydew (Grabowski & Klein, 2015; Kačániová et al., 2012)).

Eremascus, an ascomycete genus distinguishable by the formation of solitary asci, includes two species, *E. albus* and *E. fertilis* (syn. *Skoua fertilis*). Recent studies have unambiguously shown that *E. fertilis* should be excluded from the class *Eurotiomycetes*, representing a new genus, *Skoua* (Wynns, 2015). This genus is most closely related to the *Myxotrichacaeae* family, a former member of the *Eurotiomycetes* order but one that phylogenetic investigations have determined belonging to the *Leotiomycetes* order (Wang et al., 2006a,b). *S. fertilis* (M2, Figure 3.5 aa-bb) is an extremely rare species, unable to grow on high a_W media or in low solutes concentrations (Pitt & Hocking, 2009). The minimum a_W for growth and sporulation reported for *S. fertilis* is 0.77 (Rodríguez-Andrade et al., 2019; Wynns, 2015). In terms of ecology, *S. fertilis* has been isolated from low water activity substrates, including from honeycomb and on pollen in bee hives honey (Pettersson & Leong, 2011; Wynns, 2015). This specie has already been identified in honey blossom and honeydew (Rodríguez-Andrade et al., 2019), however the isolation in nectar honeys represents a new record among honeys subtracts.

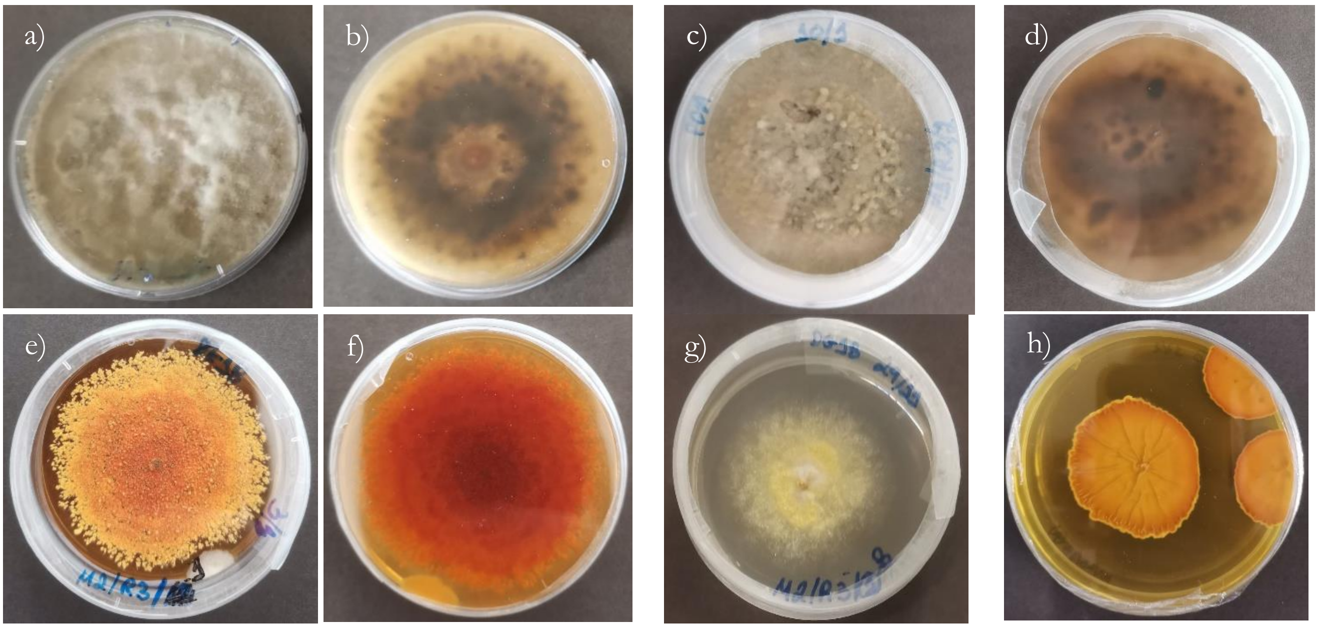
Meyerozyma yeasts are widely dispersed in the environment and inhabit plant tissues, rhizospheres, and soil (De Lima Targino et al., 2022; Moreira et al., 2020; Moreira & Vale, 2018; Nakayan et al., 2013; Peng et al., 2018). The close relatedness of *M. caribbica* with *M. guilliermondii*, an opportunistic human pathogen, raises the possibility that *M. caribbica* may also be an occasional human pathogen (Kurtzman, 2011). Apart from being organisms of clinical importance, *M. guilliermondii* and *M. caribbica* are often linked to fermented foods (Daniel et al., 2009; Hidalgo et al., 2012; Nova et al., 2009). *M. guilliermondii* is known for the production of flavor compounds in fermented food products (Wah et al., 2013). Moreover, isolates of *M. guilliermondii* and *M. caribbica* have exhibited great potential in the biological control of fungi responsible for postharvest spoilage of fruits and vegetables (Cao et al., 2013; J. R. Lima et al., 2013; Wilson L. et al., 1991). The *M. caribbica* (M2; M3, Figure 3.5 o-p) isolation, represents a new record among honey subtracts, and specifically regarding nectar honeys.

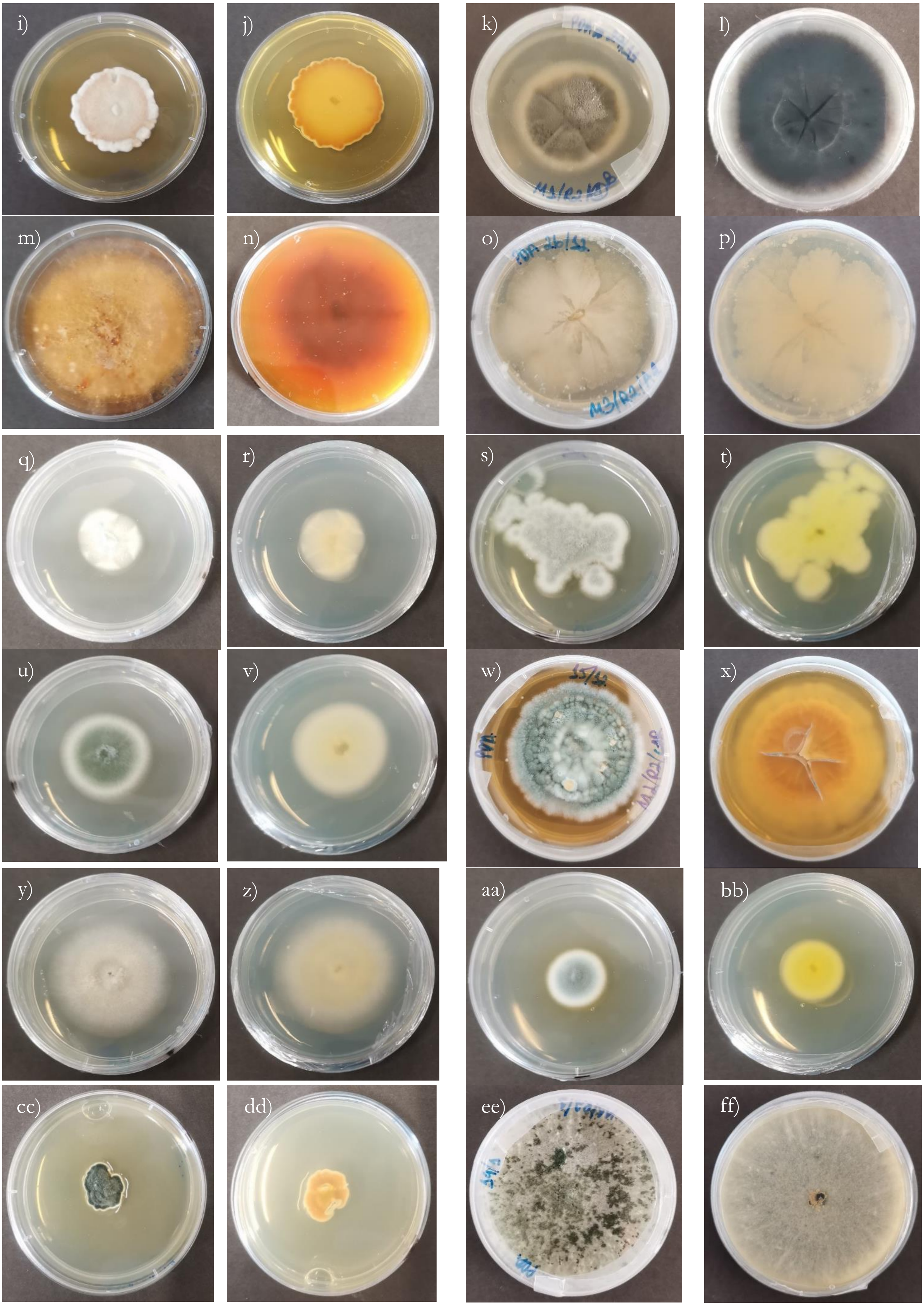
The majority of *Penicillium* species are widespread saprophytes found in almost any habitat. They can adjust to a variety of physical and chemical conditions, including various a_W, pH, temperature and redox potential. Many species are found in the nature as part of the soil mycobiome, but other species have a strong relationship with food products contributing for food spoilage (Bullerman, 2003). Species of this genus are also, sometimes, considered honey environmental contaminants (Kacániová et al., 2009). The presence of such species has already been described in most of the honey subtracts (e.g., *P. italicum* in nectar honey and honeydew (Sinacori et al., 2013) and *P. citrinum* in honey blossom, nectar honey and honeydew (Barbosa et al., 2017; Kačániová et al., 2012; Rodríguez-Andrade et al., 2019)). However, the isolation of *P. glabrum* (M1; M2, Figure 3.5 q-r), *P. janczewskii* (M3, Figure 3.5 s-t), *P. pancosmium* (M1; M2; M3, Figure 3.5 u-v) and *P. raphiae* (M2, Figure 3.5 w-x) represent new records regarding the presence of this organisms in honey subtracts.

Pseudopithomyces are similar to *Pithomyces* in terms of morphological characteristics, being saprobic/parasitic for plants (Ariyawansa et al., 2015). According to the literature, *P. rosae* (M1, Figure 3.5 y-z) has never been associated with honey subtracts, therefore the isolation of this specie emerges as a new record for honey subtracts, especially regarding nectar honeys.

The *Talaromyces* genus is well-known and among the most prevalent groups of fungi found in a range of habitats, including vegetation, air, living or decaying plants, and a wide range of food products (Nuankaew et al., 2022). The presence of species of this genus has already been described in most of the honey subtracts (e.g., *T. brunneosporus* in blossom honey and honeydew (Rodríguez-Andrade et al., 2019) and *T. scorteus* in nectar honey (Barbosa et al., 2017). However, the *T. speluncarum* isolation (M2, Figure 3.5 cc-dd) is a new record for honey subtracts, specifically concerning nectar honeys.

Numerous rhizocompetent filamentous fungus strains, found in a wide range of environments, belong to the asexually reproducing genus *Trichoderma*. In particular, this genus has the ability to adapt and thrive in different environmental conditions and have been proven effective in the sustainable management of crop diseases caused by fungi (Stracquadanio et al., 2020). In fact, *Trichoderma* species have the potential to be used as biocontrol agents for plant pathogenic fungi due to their mycoparasitic nature (e.g., the ability to attack other fungi and absorb their nutrients, in addition with the production of molecules with antifungal effect) (Brotman et al., 2010). The presence of species of this genus has already been described in most of the honey subtracts, as for example the detection of *T. hamatum* in nectar honeys (Nasser, 2004). Therefore, the isolation of *T. hamatum* (M1, Figure 3.5 ee-ff) during the course of this study is in accordance with the available literature. Nonetheless, the isolation of *T. harzianum* (M1, Figure 3.5 gg-hh) and *T. scalesiae* (M1; M3, Figure 3.5 ii-jj) represent a new record for nectar honeys.

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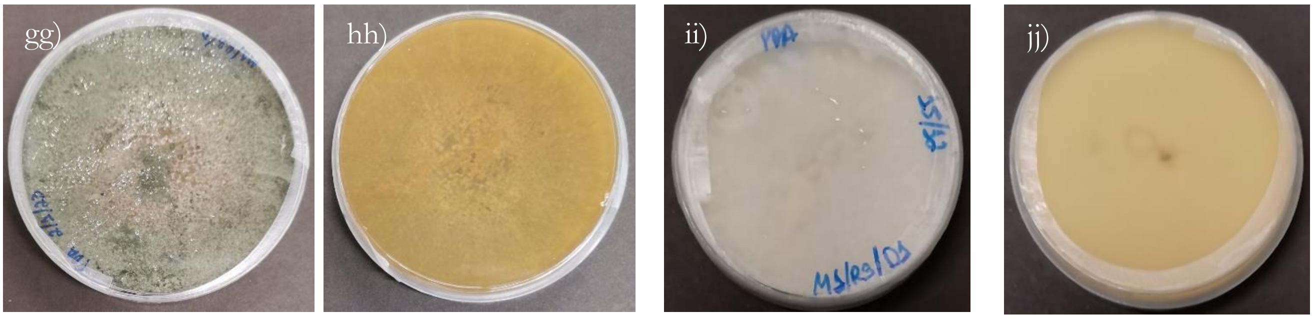


Figure 3.5 – Colony morphology of the isolates recovered from the samples on PDA and DG18 agar. The right plate in each pair is the obverse surface and the left plate is the reverse surface. **a-b)** *A. alstroemeriae* (PDA); **c-d)** *A. alternata* (PDA); **e-f)** *A. brunneus* (DG18); **g-h)** *Aspergillus* sp. (DG18); **i-j)** *B. alvei* (PDA); **k-l)** *C. cladosporioides* (PDA); **m-n)** *E. layuense* (PDA); **o-p)** *M. caribbica* (PDA); **q-r)** *P. glabrum* (PDA); **s-t)** *P. janczewskii* (PDA); **u-v)** *P. pancosmium* (PDA); **w-x)** *P. raphiae* (PDA); **y-z)** *P. rosae* (PDA); **aa-bb)** *S. fertilis* (PDA); **cc-dd)** *T. speluncarum* (PDA); **ee-ff)** *T. hamatum* (PDA); **gg-hh)** *T. harzianum* (PDA); **ii-jj)** *T. scalesiae* (PDA).

The incidence, and total number of isolated fungi, is presented in Table 3.2 – Frequency of fungi recovered from honey samples.Table 3., with the data organized by respective genera and species. The species *P. pancosmium* was the most dominant among the analyzed samples with 16 isolates (18,60%), followed by *B. alvei* and *P. rosae* with 8 isolates (9,30%). Also, with a significant number of isolates, stands out the species *M. caribbica* and *T. speluncarum* with 7 isolates (8,14%) each. On the other hand, the species with lower representation were *A. brunneus*, *P. raphiae* and *T. harzianum* with only 1 isolate (1,16%) each.

Table 3.2 – Frequency of fungi recovered from honey samples.

Genus	Species	Frequency [%]	# isolates	Frequency [%] per genus	# isolates per genus
Alternaria	*A. alstroemeriae*	5,81	5	8,14	7
	A. alternata	2,33	2		
Aspergillus	*A. brunneus*	1,16	1	3,49	3
	Aspergillus sp.	2,33	2		
Bettsia	*B. alvei*	9,30	8	9,30	8
Cladosporium	*C. cladosporioides*	5,81	5	5,81	5
Epicoccum	*E. layuense*	4,65	4	4,65	4
Meyerozyma	*M. caribbica*	8,14	7	8,14	7
Penicillium	*P. glabrum*	6,98	6	30,23	26
	P. janczewskii	3,49	3		
	P. pancosmium	18,60	16		
	P. raphiae	1,16	1		
Pseudopithomyces	*P. rosae*	9,30	8	9,30	8
Skoua	*S. fertilis*	3,49	3	3,49	3
Talaromyces	*T. speluncarum*	8,14	7	8,14	7
Trichoderma	*T. hamatum*	4,65	4	9,30	8
	T. harzianum	1,16	1		
	T. scalesiae	3,49	3		

	100,00	**86**	**100,00**	**86**

Correspondingly, in terms of genera, *Penicillium* was the most encountered in the different types of analyzed honeys, with a total of 26 isolates representing 30,23% of the found taxa. The genera *Bettsia*, *Pseudopithomyces* and *Trichoderma* were the second most frequent genera, with a total of 8 isolates (9,30%) each. The third most frequent genera were the *Alternaria*, *Meyerozyma* and *Talaromyces*, each with 7 isolates (8,14%). It is also important to highlight that the less represented genera were the *Aspergillus* and *Skoua*, just with 3 identified isolates (3,49%) each.

These results are particularly important, since *Penicillium* species are mycotoxin producers usually found in honey (Samson et al., 2010). Furthermore, the presence of *B. alvei* strains may indicate poor beehive management practices (Finola et al., 2007).

3.5 Fungal taxa by source of the nectar and geographic region

The various botanical origins provide honeys with different sensorial and nutraceutical characteristics (Ball, 2007). Knowledge of the origin of honeys is important to avoid commercial frauds, despite of honey characterization being, occasionally, a very complex process. Fungal diversity of honeys can also assist in determining the origin of the product (Gilliam, 1997; Seijo et al., 2011). Previous studies have shown that honey pH and acidity are independent of geographic origin, but related to nectar composition and botanical sources (Silva et al., 2016; Scholz et al., 2020). On the other hand, honey age, moisture and source are thought to be critical factors influencing the fungal communities of raw honey, and creating conditions for possible fermentation and spoilage (Balzan et al., 2020a). This is particularly relevant when compared with other factors like pH, a_W and geographical origin of the honey, classified as minor factors, which in turn have a lower influence on the fungal communities (Xiong et al., 2023). On the other hand, some other authors have stated that the geographical region may be an important factor influencing microbial communities (fungi and bacteria) in bee products (Disayathanoowat et al., 2020). For the species occasionally found, a correlation with the botanical or geographical origin could not be found, instead, only possible environmental contaminations could be assumed (Xiong et al., 2023).

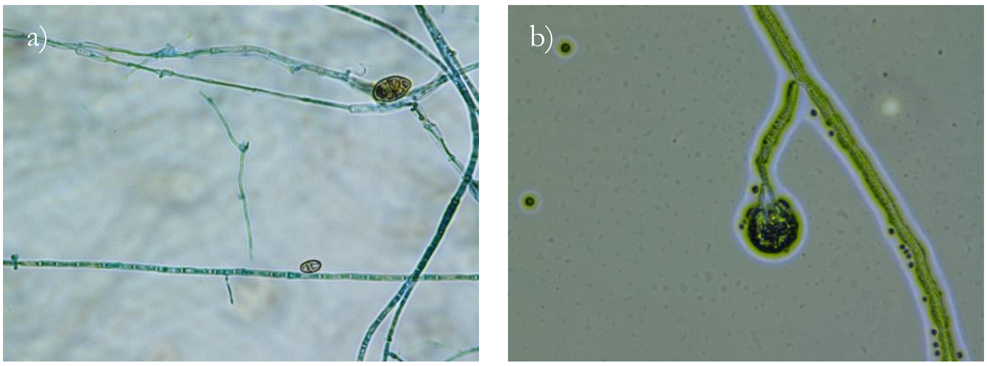
The distribution of the microorganisms isolated regarding the geographical region is reported in Table .3. The species *P. pancosmium* was the sole present in all the geographical regions considered. The species *M. caribbica*, *P. glabrum* and *T. scalesiae* showed a similar distribution, being present in two of the studied regions. The results also showed that *A. brunneus*, *Aspergillus* sp. (possible new species in subgenus *Aspergillus*; section *Aspergillus*: series *Aspergillus* (Houbraken et al., 2020)), *B. alvei*, *P. raphiae*, *S. fertilis* and *T. speluncarum* are only present in one region, in this case the Region of Beira Baixa. The same also occurs for *A. alstroemeriae*, *A. alternata*, *C.*

cladosporioides, *E. layuense*, *P. rosae*, *T. hamatum* and *T. harzianum* only isolated for the Region of Setúbal.

Table 3.3 – Distribution of the microorganisms throughout honeys of different botanical origin and geographical region.

Microorganisms	Source of the nectar		Geographical region		
	Lavandula spp.	*Eucalyptus* spp.	Region of Beira Baixa	Region of Beira Litoral	Region of Setúbal
A. alstroemeriae	×				×
A. alternata	×				×
A. brunneus	×		×		
***Aspergillus* sp.**	×		×		
B. alvei	×		×		
C. cladosporioides	×				×
E. layuense	×				×
M. caribbica	×	×	×	×	
P. glabrum	×		×		×
P. janczewskii		×		×	
P. pancosmium	×	×	×	×	×
P. raphiae	×		×		
P. rosae	×				×
S. fertilis	×		×		
T. speluncarum	×		×		
T. hamatum	×				×
T. harzianum	×				×
T. scalesiae	×	×		×	×

The isolates were also examined using the microscopic methods, enabling a more thorough examination of each organism and the visualization of some of the most distinctive structural features (e.g., hyphae, conidiophores and conidia) (Figure .6).



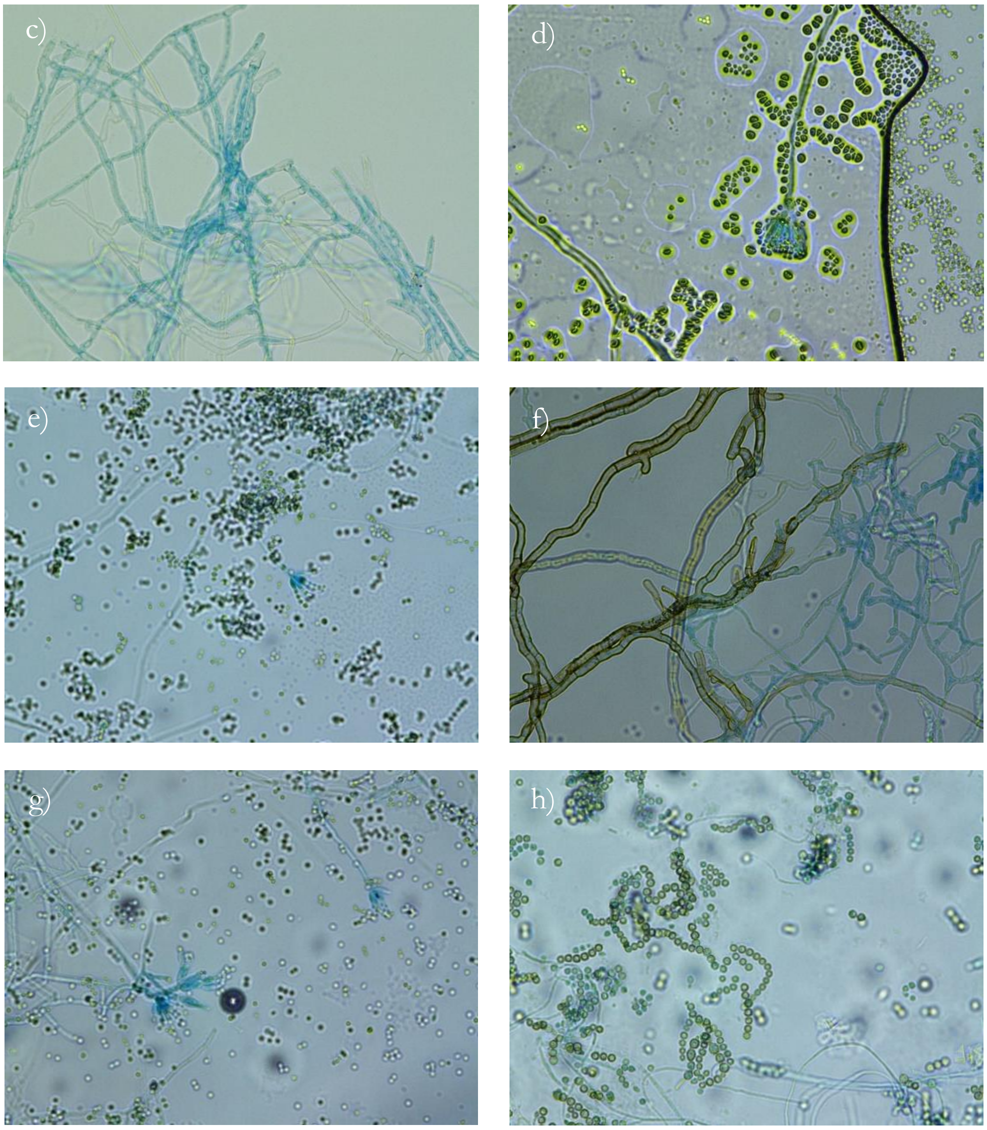


Figure 3.6 – Microscopic observation of selected structures of the recovered filamentous fungi under 40X magnification. **a)** *A. alstroemeriae* (conidia); **b)** *B. alvei* (spore cysts containing spherical ascospores); **c)** *E. layuense* (hyphae smooth, branched, septate and hyaline); **d)** *P. glabrum* (conidiophores and conidia); **e)** *P. janczewskii* (conidiophores and conidia); **f)** *P. rosae* (septate hyphae); **g)** *S. fertilis* (conidiophores and conidia); **h)** *T. speluncarum* (chains of globose conidia).

3.6 Analysis of High Throughput Sequencing data

The present study also aimed to characterize the considered raw monofloral Portuguese honeys by means of metabarcoding, contributing to understand putative differences in the fungal communities across distinct geographical regions and honey botanical sources. It is believed that this characterization can also be helpful to evaluate potential threats and assist decision-making processes aiming to improve and maintain this critical industry in the country. Moreover, considering the culture approach also conducted, this study also allows for a comparison of both methods when applied in these substrates.

The relationship between samples, defined by S1 (M1 sample), S2 (M2 sample) and S3 (M3 sample), was studied and is represented in Figure .7, through a Venn diagram displaying the shared OTUs. Among the OTUs detected in this study, 6,39% (20) appeared in all the samples, 29,39% (92) appeared only in S2 sample, 16,29% (51) appeared only in S1 sample, and 25,88% (81) appeared only in S3 sample. The samples S2 and S3 (*Lavandula* spp. vs *Eucalyptus* spp.) had the higher number of shared OTUs (50) when compared with samples S1 and S2 (10) or samples S1 and S3 (9) which presented relatively similar OTU overlaps.

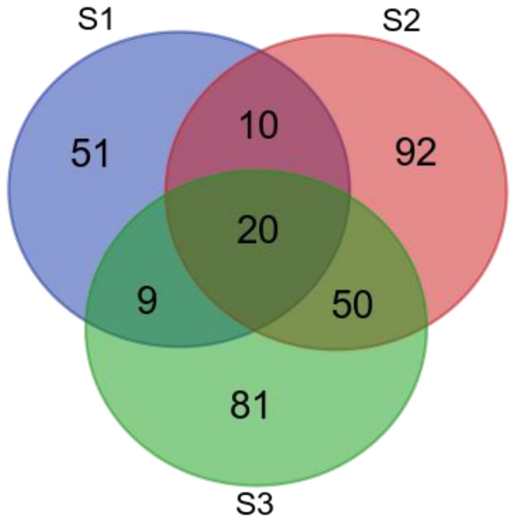


Figure 3.7 – Venn diagram displaying the shared OTUs between samples.

3.7 Metabarcoding data analysis

The microbiome analysis was achieved by sequencing of the ITS2 subregion with the Illumina MiSeq® sequencing platform. To the best of our knowledge, this is one of the first works using amplicon metagenomics to study Portuguese raw honeys. Table .4 highlights the obtained metabarcoding statistical data and the diversity indexes calculated for each taxonomic group in the different samples considered.

Table 3.4 – Metabarcoding statistical data and biodiversity indexes.

Sample	nseqs	coverage	Chao1	sobs	shannon	shannoneven	simpson	invsimpson	simpsoneven
S1	104998	0,999924	97	90	3,41568	0,759072	0,055966	17,868085	0,198534
S2	180946	0,999928	181,75	172	2,016933	0,391828	0,248937	4,017079	0,023355
S3	186798	0,999882	185,66667	160	2,558154	0,504053	0,151618	6,595527	0,041222

By examining the obtained Chao1 richness values, sobs and the coverage values, it can be verified that the fungal diversity was successfully analyzed (Table .4). Overall, 472742 reads were obtained, with the OTU number determined for each sample being 90 for sample S1, 172 for sample S2 and 160 for sample S3. In total, 313 OTUs were detected. In terms of individual values for estimated richness and diversity, the sample S2 (*Lavandula* spp. from the central Portuguese region) had the lowest values between all samples, pointing to the possible presence of more stringent conditions for their development in this raw honey (e.g., higher acidity, honey age, lower pH, titratable acidity levels, brix, higher presence of antimicrobial compounds and lower moisture content) (Balzan et al. 2020; Jacinto-Castillo et al., 2022; Xiong et al. 2023). When comparing the two *Lavandula* spp. honey samples, the one originating from the South of Portugal (S1) exhibited a higher diversity than the one from the Center of Portugal (S2). On the other hand, when considering the two raw honeys from the central regions of Portugal (S2 and S3; *Lavandula* spp. *vs Eucalyptus* spp.), they had the highest number of shared OTUs (50), suggesting an important impact regarding the original substrate geographical origin on the mycobiome. Nonetheless, due to the high number of OTUs solely present in a single individual sample, and through the analysis of the Shannon and Simpson diversity values, it is clear that these honeys also harbor diverse and distinct microbiomes (Xiong et al., 2023). Moreover, the results obtained also highlight a tendency for the presence of some dominant and diverse Fungal taxa across the studied samples.

3.8 Biodiversity characteristics - data analysis

The metabarcoding analysis regarding the biodiversity of each sample (Figure .8), revealed some of the dominant genera unidentified by the culture dependent methods. The genera *Malassezia* (19,5%), *Zygosaccharomyces* (15,4%), unclassified Ascomycota (8,6%), *Bettsia* (6,4%), *Trametes* (4,5%) and *Cladosporium* (4,4%) were dominant in sample S1; *Bettsia* (44,2%), unclassified Ascomycota (25,2%), *Metschnikowia* (16,7%) and *Talaromyces* (5,4%) were dominant in sample S2; and *Metschnikowia* (45,5%), *Bettsia* (13,2%), unclassified Ascomycota (10,6%), *Yarrowia* (8,6%), *Aspergillus* (4,8%), *Candida* (4,5%) and *Starmarella* (2,8%) were dominant in sample S3. Overall, for the mycobiota of the studied raw honeys, their populations were largely dominated by *Malassezia*, *Zygosaccharomyces*, *Bettsia*, *Trametes*, *Cladosporium*, *Metschnikowia*, *Talaromyces*, *Yarrowia*, *Aspergillus*, *Candida* and *Starmarella*. The detected yeasts (*Candida*, *Malassezia*, *Metschnikowia*, *Starmarella* and *Zygosaccharomyces*) have been commonly detected on honeys in the past, with some known to be extremophilic and able to endure in the typical conditions found in these substrates (e.g., *Zygosaccharomyces*) (Balzan et al., 2020b; Grabowski & Klein, 2015; Jacinto-Castillo et al., 2022; Olaitan et al., 2007; Pozo & Jacquemyn, 2019; Silva et al., 2017; Sinacori et al., 2013; Xiong et al., 2023), and some species of *Candida* and *Malassezia* able to pose human health problems (Ciurea et al., 2020; Hobi et al., 2022). On the other hand, *Metschnikowia* has been hypothesized to originate from nectars, thus allowing them to be transmitted from plants to honeybee products (Xiong et al., 2023). The genus *Yarrowia* contains oleaginous yeasts, that can use unusual carbon sources (e.g., hydrocarbons) present in environments with high contents of lipids (Flores & Gancedo, 2005; Nicaud, 2012). Complementarily, the main filamentous fungi detected (*Aspergillus*, *Bettsia*, *Cladosporium*, *Trametes* and *Talaromyces*) contain many xerophilic species, thus possessing the necessary characteristics to survive in these substrates (cet al., 2019). *Aspergillus* are often reported associated with honeys and, being ubiquitous fungi, are able to be transported by bees (Balzan et al., 2020b; Jacinto-Castillo et al., 2022). Nonetheless, some *Aspergillus* species are bee pathogens (Becchimanzi & Nicoletti, 2022), human pathogens (Vuong et al., 2023), cause food spoilage (Houbraken et al., 2020), considered honey environmental contaminants (Kacániová et al., 2009) and can also cause stonebrood disease (as previously mentioned) (Felsöciová et al., 2012). *Bettsia* contains obligate xerophiles (*B. alvei*) being commonly reported in honey, as well as in hives, honeycombs, pollens and bee waxes (Rodríguez-Andrade et al., 2019). *B. alvei* can cause pollen to become moldy, pointing out poor beehive management practices and causing concern when detected in high levels (Felsöciová et al., 2012). *Cladosporium* species are cosmopolitan, being found in honey from the surrounding environments (Jacinto-Castillo et al., 2022). In addition, some species of *Cladosporium* can cause human health problems (Sandoval-Denis et al., 2015). *Trametes* often serve as food for animals and their presence is also likely environmental. As for *Talaromyces*, they have also been commonly found associated with honeys (Rodríguez-Andrade et al., 2019), can produce heat-resistant ascospores contributing to spoilage of processed foods (Houbraken et al., 2020), and some species may also cause human health problems (Mahajan, 2021).

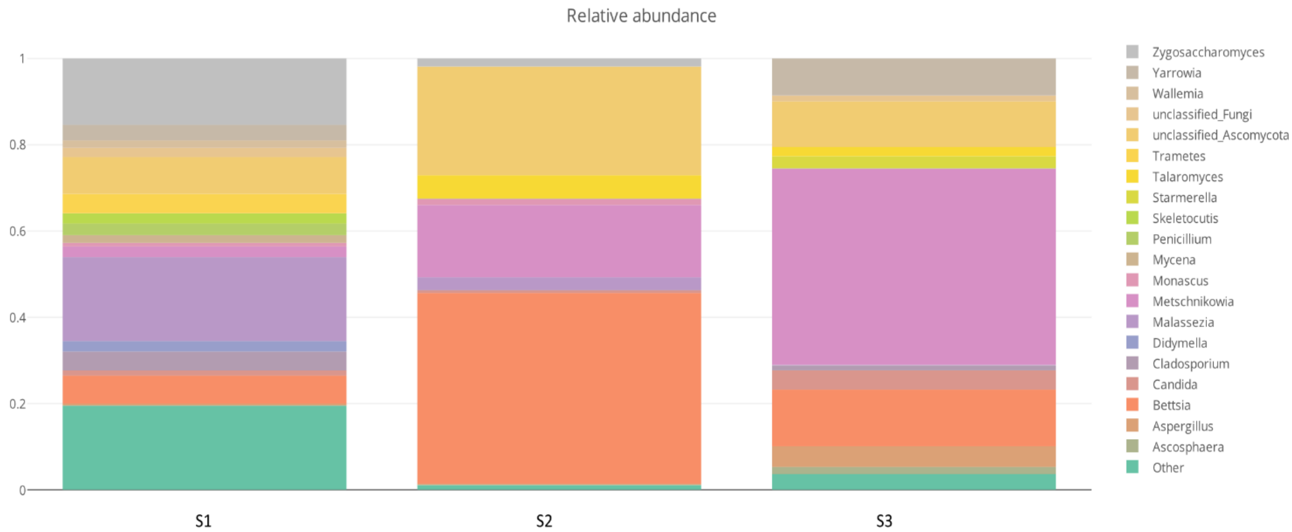


Figure 3.8 – Relative abundance of the top 20 fungal genus found in the three considered raw honey samples.

3.9 Data analysis - general overview

To date, only eighteen fungal species have been found across three main studies (Carvalho et al., 2010; Magyar et al., 2005; Martins et al., 2003) related with Portuguese honeys. The species identified in this work increase the number of microorganisms (filamentous fungi and yeasts) regarding this subject, with the addition of eighteen fungal species to the knowledge of this thematic. The present study also made it possible to increase the number of species identified in nectar honeys with twelve new additions regarding filamentous fungi (divided in eight genera), namely *A. alstroemeriae*, *A. brunneus*, *E. layuense*, *M. caribbica*, *P. glabrum*, *P. janczewskii*, *P. pancosmium*, *P. raphiae*, *P. rosae*, *T. speluncarum*, *T. harzianum* and *T. scalesiae*. The results also confirm the presence of some species related with honey subtracts (e.g., *A. alternata*, *Aspergillus* sp., *B. alvei*, *C. cladosporioides*, *S. fertilis* and *T. hamatum*. In terms of yeasts, this study allowed to identify the species *M. caribbica*, belonging to the *M. guilliermondii* species complex, commonly present in nature and isolated from a variety of sources (De Marco et al., 2018).

It is also important to highlight that the enumeration and isolation of viable osmophilic yeasts and xerophilic fungi (with an a_W lesser or equal to 0.95) was achieved through the use of DG18 media (Black, 2020; Hocking & Pitt, 1980). The reduction of a_W in this medium, achieved by the addition of glycerol (at approximately 18%), is necessary given that many yeasts and molds require a low water activity to enhance their growth and colony development. In addition, dichloran is added to limit the spread of some fast-growing fungal colonies, limiting their diameter (Black, 2020; Himedia, 2020). For the case of PDA, the extract from potato and dextrose, with a typical a_W of 0.98 (Schultz, 2016), provides a nutritionally rich base that encourages mold sporulation and pigment production.

Despite the stressing conditions of honey, the microbiological analyses revealed the presence of several fungal groups. The results suggest that the most frequent fungal taxa in floral honeys belong to ubiquitous species of fungi (*Alternaria*, *Bettsia*, *Penicillium*, *Pseudopithomyces* and *Talaromyces*), as well as several xerotolerant species of *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* (Grabowski & Klein, 2015; Kacániová et al., 2009; Kačániová et al., 2012; Rodríguez-Andrade et al., 2019; Sinacori et al., 2013; Snowdon & Cliver, 1996; Pettersson & Leong, 2011), common contaminants of honey isolated from different insects, including honeybees, hives, bee products, pollen grains, and soil (Gilliam, 1997; Grabowski & Klein, 2015; Kačániová et al., 2009). On the other hand, less frequent fungi are apparently more specific. Some fungi found in honeys are considered to be non-specific to the host, being common saprotrophs, e.g., *Alternaria* and *Cladosporium*, as saprophytic genera, feeding on sweet products and honeydew (Magyar et al., 2005, 2016; Seijo et al., 2011).

Many species in the genera *Aspergillus* and *Penicillium* can be described as moderate xerophiles, particularly those that cause spoilage in stored commodities (grains, nuts, and spices), baked goods and other intermediate a_W foods. These fungi are also of significance in the indoor environment, as they can grow on building materials and cause mildew on wood, clothing and leather (Pettersson & Leong, 2011). The slow-growing xerophiles, e.g., *Bettsia*, *Eremascus* and *Skoua* (Pettersson & Leong, 2011) can live in the bee habitat because it is a temporally static microenvironment that offers low a_W substrates, such as honey, bee bread, and pollen supplies (Wynns, 2015). In addition, *Eremascus* and *Skoua*, due to their physiological needs as xerophiles, may require bee-mediated dispersal more than other species (Pettersson & Leong, 2011).

The possible presence of mycotoxigenic fungi in foods, and rational decisions on the status of foods suspected to contain mycotoxins, are ever present problems in the food industry around the world (Frisvad et al., 2006). Therefore, it is important their identification (Felsöciová et al., 2012). Numerous fungal species, mainly *Aspergillus* and *Penicillium* species, due to some environmental factors such as a_W below 0.70 are potential producers of mycotoxins, making honey less desirable for consumers (Mannaa & Kim, 2017). These species in particular can produce the mycotoxin patulin as a secondary metabolite, generally associated with fungal infected food materials (El-Sayed et al., 2022). Although the majority of secondary metabolites are rather benign, *A. alternata* and other *Alternaria* species are capable of creating dibenzo-pyrone mycotoxins (e.g., alternariol, altenuene, isoaltenuene, and altenuisol) (Chu, 2003). The anamorph and filamentous xerophilic species *P. janczewskii* is also capable of producing griseofulvin, a mycotoxin active against various species of dermatophytic fungus from the genera *Microsporum*, *Trychophyton*, and *Epidermophyton* (De Carli & Larizza, 1988; Pettersson & Leong, 2011). Mycotoxins, however, are not always produced under the same conditions that are required for fungal growth (Barkai-Golan & Paster, 2008), therefore they are not always present when these microorganisms first appear. It needs ideal conditions such as high water activity, and the presence of sugars and organic acids capable of reducing pH (Silva et al., 2017).

This work also analyzed the diversity and community composition of filamentous fungi and yeasts in nectar honeys using both a culture-dependent and independent approach. Traditional taxonomy has employed morphology-based species identification. Culture-specific traits and the study of asexual morphs are crucial components of identification and characterization. Cultivation allows the isolation and characterization of individual microbial strains, provides pure cultures for detailed studies, including identification, phenotypic characterization, and physiological analysis, and can be used to test the susceptibility of microorganisms to antimicrobial agents. However, only a small fraction of microorganisms in a sample can be cultured using standard techniques leading to a biased representation of the microbial community. Some microorganisms may have specific growth requirements that are difficult to replicate in the laboratory, resulting in their underrepresentation or failure to be detected (Dissanayake et al., 2018). To overcome the shortcomings of the culture-dependent approach, culture-independent approaches have been suggested as an alternative (Gomez et al., 2017; Hoppe et al., 2016; Peršoh, 2015; Zapka et al., 2017). As a result, metagenomics and NGS techniques are crucial to environmental sequencing research (Wijayawardene et al., 2021). They can detect and identify a broader range of microorganisms, including those that are difficult, or impossible, to culture using traditional methods, providing a more comprehensive understanding of the microbial community composition and functional potential. In addition, they offer a high-throughput approach, allowing for the analysis of a large number of samples simultaneously (Jiang et al., 2022). In terms of limitations, this technique does not provide viable isolates for further characterization and study, may lack resolution at the species level (often due to their short-fragment target nature), especially when using broad-target genes or shotgun metagenomic sequencing, and the presence of non-viable or dormant microorganisms can lead to false-positive results (Jiang et al., 2022; Liu et al., 2022).

Nevertheless, this work demonstrates that the two applied methods are complementary, since species not identified by one method are identified by the other (e.g., *B. alvei* identified in only one sample through culture-dependent methods, and in all samples when using culture-independent methods). NGS technologies, together with general culturing methods, allow synchronized exploration of a more complete picture of the fungal communities in honey substrates. Using a predetermined similarity criterion based on the intra- and inter-specific variation of the isolates, this idea is applicable when fungal genera are identified by sequencing the pure cultures produced using the culture-dependent approach and comparing them to OTUs obtained using the culture-independent method (Dissanayake et al., 2018).

4 CONCLUSION

Honey retains a natural product image, with the increasing trend in consumption being attributed to a new lifestyle, demanding more natural foods and associated beneficial effects. However, the quality of this product could be compromised by poor hygiene practices during harvest and extraction, as well as storage time and conditions. The presence of undesired microorganisms, able to develop and grow on the surface or encroaching into their matrices, can also provide a clear indication of honey safety. In fact, honey microbiota is a complex matrix containing ecological information about the host microenvironment, the hive pathosphere, and the honeybee hologenome. However, the presence of pathogenic fungi does not necessarily mean that beehives are infected. Certain environmental factors are required for these foodborne pathogens to manifest, with most of them surviving in honey solely as dormant spores. Despite this, honeys must obey rigorous ecological and natural principles, intended to improve its safety and quality. It is known that this product may occasionally be impacted by fungi during the various steps of the production process and, therefore, get contaminated or lose some of its nutritional value due to fungal growth.

Microorganisms can get into honey primarily from the digestive tract of bees, honeycomb, pollen, nectar, dust, air, or soil, and, hence, difficult to control. The so-called "secondary microbial contamination" of honey during processing and storage can occur depending on storage conditions or improper technological procedures (beehives, beekeepers, buildings, inventory, equipment and storage vessel). Taking into consideration possible cross-contaminations, microbial control can be exerted by following good beekeeping principles and manufacturing practices (training and handling). Honey microbiological traits, inherent to its quality and safety, provide information about production faults (contaminated environments) and violations of good beekeeping principles and manufacturing practices. Therefore, the interest towards the microbial composition of commercial honeys is of paramount importance.

Knowing the fungal diversity is important for the ecosystem and food supply characterization, with some studies highlighting the presence of certain fungi in honey, as a way to understand and potentially verify the botanical and the geographical source of honeys produced in different countries. Identifying the fungal species present in honey samples is a helpful tool when trying to identify the honey's botanical source or to validate its origin. Although quantitative data (presence or absence of fungi) can be helpful, qualitative data (needing additional research) is even more valuable. The richness and diversity of the honey flora in Portugal, both from wild species and cultivated plants such as eucalyptus (*Eucalyptus* spp.), means that there is a great diversity of monofloral honeys. These typically offer a unique sensory experience, and in certain cases are associated with therapeutic effects according to their botanical origin.

In this work, and making use of culture-dependent methods, a total of 79 filamentous fungi and 7 yeasts were recorded in the analyzed samples, distributed between the two employed culture media. Regarding the source of the nectar, the presence of fungal species is more predominant in the samples of *Lavandula* spp. (74 isolates) when compared with the *Eucalyptus* spp. samples (12 isolates). From a molecular point of view, the isolated mycobiota accounted for *A. alstroemeriae*, *A. alternata*, *A. brunneus*, *Aspergillus* sp., *B. alvei*, *C. cladosporioides*, *E. layuense*, *M. caribbica*, *P. glabrum*, *P. janczewskii*, *P. pancosmium*, *P. raphiae*, *P. rosae*, *S. fertilis*, *T. speluncarum*, *T. hamatum*, *T. harzianum* and *T. scalesiae*. The species *P. pancosmium* (18,60%), *B. alvei* (9,30%) and *P. rosae* (9,30%) were those that displayed a higher frequency among the samples in study. Furthermore, the genera *Alternaria*, *Bettsia*, *Penicillium*, *Pseudopithomyces*, *Talaromyces* and *Trichoderma* were the taxa of filamentous fungi most frequently found in the honey, while *Meyerozyma* was the only yeast genus found. The two most significant pathogenic fungus for honeybees, *Aspergillus flavus* and *Ascosphaera apis*, were not identified throughout the course of this investigation. Nonetheless, the identified microorganisms contributed for the enlightenment of the fungal species in Portuguese honeys, allowing to increase the list of species identified so far by adding eighteen new entries.

Almost all honeys, regardless of its provenance, present common species including a variety of xerotolerant and xerophilic fungus (e.g., *B. alvei*). Such fungi can vary qualitatively and quantitatively depending on, among other physicochemical and biological parameters, the geographical origin, plant species and honey water activity. *P. pancosmium* was the only species present in all the geographic regions considered. The species *M. caribbica*, *P. glabrum* and *T. scalesiae* showed a similar distribution being present in two of the regions studied (Region of Beira Baixa and Region of Beira

Litoral). The majority of the species identified came from the M1 sample, collected in the south region of Portugal (Region of Setúbal), representing 34,88% of the total number of isolates. Despite the majority of the species identified coming from the south region, the conducted study allowed to expand the knowledge on monofloral honey originating from different nectar sources and geographical areas. The identification of microbial species, linked to floral honeys, supports the notion that honey holds highly selective stressors. As is the case with the species detected in this work, the origin of some of these fungi, tied to the honeybee lifestyle, is yet unknown. In the latter scenario, aphids and flowers may both be significant sources of these fungus. At the same time, the findings of this work demonstrate that the honey consumed in Portugal is a rich source of important mycotoxin producers, particularly those belonging to the *Penicillium* genus.

This work also attempted to provide a snapshot of the characteristics of the monofloral honeys using metabarcoding approaches, a culture-independent method. This NGS based technology allowed to determine that each honey harbors diverse and distinct microbiomes. The *Lavandula* spp. honey from the South of Portugal (M1) has a higher diversity and evenness than the *Lavandula* spp. (M2) and *Eucalyptus* spp. (M3) from the Central Portuguese region. Some dominant fungal taxa are present across the various studied honeys and multiple problematic taxa, in all stages of honey production, have been identified, including organisms potentially affecting bee health, human health and food spoilage, inferring poor beehive management practices. In summary, this work demonstrated that the two applied methods should be considered of complementary nature.

NGS technologies together with general culturing methods allow synchronized exploration of a more complete picture of the fungal communities in honey substrates. Thus, in the future, new analyses should be used alongside more specialized culture media and next generation technologies in widespread nectar sources to enable a continuous characterization of the mycobiota of Portuguese honey. Based on this study, and previous reports investigating and monitoring honey mycobiota, the screening of potential pathogenic microorganisms should be largely implemented.

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