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Mould spoilage of foods and beverages: Using the right methodology

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ABSTRACT

Fungal spoilage of products manufactured by the food and beverage industry imposes significant annual global revenue losses. Mould spoilage can also be a food safety issue due to the production of mycotoxins by these moulds. To prevent mould spoilage, it is essential that the associated mycobiota be adequately isolated and accurately identified. The main fungal groups associated with spoilage are the xerophilic, heat-resistant, preservative-resistant, anaerobic and psychrophilic fungi. To assess mould spoilage, the appropriate methodology and media must be used. While classic mycological detection methods can detect a broad range of fungi using well validated protocols, they are time consuming and results can take days or even weeks. New molecular detection methods are faster but require good DNA isolation techniques, expensive equipment and may detect viable and non-viable fungi that probably will not spoil a specific product. Although there is no complete and easy method for the detection of fungi in food it is important to be aware of the limitation of the methodology. More research is needed on the development of methods of detection and identification that are both faster and highly sensitive.

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1. Introduction

Fungal spoilage of foods and beverages imposes significant annual global revenue losses for the food and beverage industries. Mould spoilage can also be a food safety issue due to the production of mycotoxins or allergens by these moulds. To avoid or reduce mould spoilage, several hurdles can be used: (1) reducing the water activity of the food, (2) thermal processing, (3) addition of preservatives, (4) reduction of oxygen in the packaging using vacuum, oxygen scavengers or modified atmosphere packaging (MAP), and (5) refrigerated storage. However, the addition of each of these hurdles will be selecting for a different group of spoilage fungi (Fig. 1). The use of two or more hurdles will reduce the number of moulds that can spoil the product. This is called the associated mycobiota, typically comprised of several mould species, many of which are known to promote accelerated product spoilage (Filtenborg et al. 2004). The fungi groups associated with fungal spoilage are: (1) xerophilic fungi that can grow at water activities (a_w) below 0.85 and spoil low and intermediate a_w foods; (2) heat-resistant fungi that survive pasteurization and baking, spoiling heat-processed products; (3) preservative-resistant fungi that can grow in the presence of preservatives such as sorbates or benzoates and spoil beverages and foods that are preserved; (4) anaerobic fungi that can grow under very low oxygen concentration and spoil vacuum or modified atmosphere packaged foods and (5) psychrophilic and psychrotolerant fungi that can grow under refrigeration and spoil refrigerated foods and beverages. A few heat-resistant fungi such as *Paecilomyces variotii* are also preservative-resistant. Some of the preservative-resistant moulds such as *Penicillium roqueforti* can grow at low oxygen concentrations as well as under refrigeration. To prevent mould spoilage, it is essential that the associated mycobiota be adequately isolated and accurately identified. The appropriate methodology and media must be used. The high-water activity media such as malt extract agar (MEA) or dichloran rose bengal chloramphenicol (DRBC) agar used for species which grow at high water activity will not be able to recover many of the moderate xerophilic moulds due to growth competition by moulds that grow faster at these water activities (Hocking and Pitt, 1980; Hocking, 1992). Extreme xerophiles such as *Xeromyces bisporus* need media containing at least 50% glucose (Pitt and Hocking, 2009). To isolate heat-resistant moulds from ingredients and environmental samples, a heat-shock step must be used (Rico-Munoz et al., 2015). Preservative-resistant fungi can be isolated using a selective media containing preservatives such as acetic acid (Pitt and Hocking, 2009).

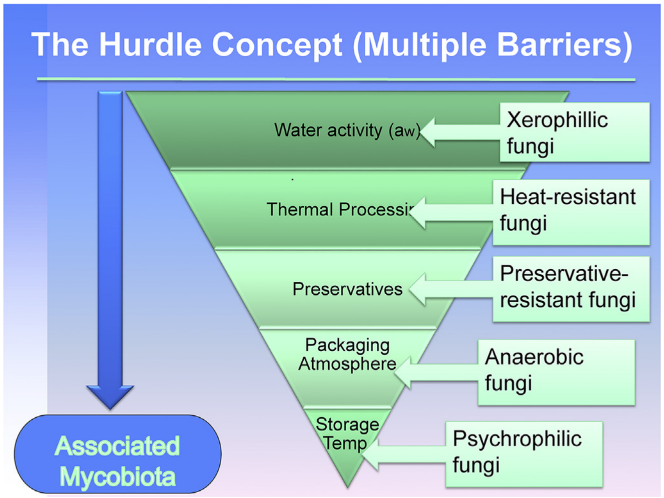

Fig. 1. To avoid or reduce mould spoilage, several hurdles can be used: (1) reducing the water activity of the food, (2) thermal processing, (3) addition of preservatives, (4) reduction of oxygen in the packaging using vacuum, oxygen scavengers or modified atmosphere packaging (MAP), and (5) refrigerated storage. However, the addition of each of these hurdles will be selecting for a different group of spoilage fungi – the associated mycobiota.

While classic mycological detection methods can detect a broad range of fungi using well validated protocols, they are time consuming, required skilled personnel and some of the methods have low sensitivity. New molecular detection methods are faster but require good DNA isolation techniques, expensive equipment and may detect fungi that would not spoil a specific product. For the identification and other characterization of isolates, reference data (sequences, profiles) are needed and those are sometimes not available. Although there is no complete and easy method for the detection of fungi in food it is important to be aware of the limitation of the methodology.

This overview emphasizes the importance of using the right methodology for the detection, enumeration, and prevention of potential fungal spoilage of food and beverage products. It also compares classic to modern methodology for the detection of spoilage fungi.

2. Conventional methodology for the detection and enumeration of spoilage fungi

Conventional or classic methods have the advantage of detecting a wide variety of fungi using conventional media and methodology that have been validated and recognized as standard methods. There are methods specific for each group of spoilage fungi. Samson et al. (2004b, 2010) described the most common methods for the detection, isolation and characterization of food-borne fungi. In the case of direct microscopical examination of fungi growing on spoiled foods, a minimally trained technician can determine the fungal genus in a short time. However, most classical methods are time consuming requiring at least 5 day as well as trained laboratory technicians. To investigate food and beverage mould spoilage cases, the right methodology and media must be used. In this section, the right methodology used for the detection of xerophilic, heat-resistant, preservative-resistant, anaerobic and psychrophilic fungi will be described.

2.1. Xerophilic fungi

Xerophilic fungi are defined by the ability to grow under conditions of reduced water activity, i.e. to complete their life cycles on substrates that have been dried or concentrated, in the presence of high levels of soluble solids such as salts or sugars (Pitt and

Hocking, 2009). They can be subdivided among two groups (Hocking, 2001; Pitt and Hocking, 2009). The first contains those that are capable of growing at or below 0.85 a_W values, generally known as moderate xerophiles. The second group, known as extreme xerophiles, contains filamentous fungi that either cannot grow at high a_W (i.e. above about 0.96–0.97 a_W) or grow very slowly and consequently compete very poorly in high a_W environments. Media used in the isolation of fungi from both groups have different requirements of a_W (Hocking, 2001; Pitt and Hocking, 2009). There are several excellent references with descriptions of these fungi (Pitt and Hocking, 2009; Samson et al., 2010). The following methods should be used not only for intermediate and low water activity foods but also for pet foods (Mello et al., 2015).

2.1.1. Moderate xerophiles

Many important food spoilage fungi can be described as "moderate xerophiles" such as *Penicillium* species, some *Aspergillus* species (including species with eurotium-morph) and *Paecilomyces variotii*. The most common *Aspergillus* species (with eurotium-morph) are *A. montevidensis* (=*A. amstelodami*), *A. chevalieri* (=*Eurotium chevalieri*), *A. pseudoglaucus* (=*Eurotium repens*), *A. ruber* (=*Eurotium rubrum*) and *A. glaucus* (=*Eurotium herbariorum*) (Chen et al., 2017; Pitt and Hocking, 2009; Samson et al., 2010). They produce mildly heat-resistant ascospores that, under some conditions, can survive heating at 70–75 °C for several minutes. These species are widely associated with deterioration of stored commodities (cereal grains, oilseeds, nuts, spices), flour baked goods (bread, fruit cakes), pet food products, dried and fermented meat and fish products, cheeses, and high sugar products such as confectionery, dried fruit, jams and conserves (Hocking, 2001). Only a few *Penicillium* species are capable of growth below 0.80 a_W and among these are *P. brevicompactum, P. chrysogenum, P. implicatum* and other species such as *P. cinnamopurpureum* (= *Eupenicillium cinnamonpurpureum*) (Hocking and Pitt, 1979; Pitt and Hocking, 1997, 2009). *Aspergillus* species are both tolerant of elevated temperature and reduced a_W, and many important food spoilage species produce mycotoxins (Hocking, 2001). Some of the most xerophilic species are *A. restrictus* and *A. penicillioides*. The species most tolerant to low oxygen tensions (0.45% oxygen) is *A. candidus*. Among the mycotoxigenic fungi, *A. flavus* and *A. parasiticus* are capable of growth at a minimum a_W of 0.80 at 30 °C. In the group of the black aspergilli, *A. niger* can grow down to 0.77 a_W at 35 °C. *Aspergillus ochraceus* (often misidentified and probably should be identified as *A. westerdijkiae*) can also grow at 0.77 a_W at 25 °C while *A. versicolor* has a minimum a_W for growth of 0.78 and 0.80 (Hocking, 2001).

Dichloran 18% glycerol agar (DG18) (Hocking and Pitt, 1980) is the media of choice for the enumeration of xerophilic fungi from reduced moisture foods (Åkerstrand, 1992; Corry et al., 2006; Frändberg and Olsen, 1999; Hocking, 1992; Pitt and Hocking, 1997; Samson et al., 1992, 2010; Ryu and Wolf-Hall, 2015). In a collaborative study, it was found to be the most satisfactory media for enumeration of fungi in reduced a_W foods (Hocking, 1992). DG18 is now widely used as an effective general-purpose medium both for foods and for sampling of indoor air (Hoekstra et al., 2000). Its water activity (0.955) reduces interference from both bacteria and rapidly growing fungi.

For spoiled foods of intermediate a_W, direct plating placing small pieces (3–6 per plate) without surface disinfection onto DG18 is recommended. Plates should be incubated upright at 25 °C for 7 days (Pitt and Hocking, 1997, 2009). For quantitative purposes, food samples can be diluted (1:10) before plating. Soaking for 30–60 min in 0.1% aqueous peptone solution before homogenizing is recommended (Hocking et al., 2006; Pitt and Hocking, 1997, 2009). Spread plates are recommended. Inocula should be 0.1 ml per plate, spread with a sterile, bent glass rod. Plates should be incubated upright at 25 °C for 7 days. A higher temperature, e.g. 30 °C, is suitable in tropical regions (Pitt and Hocking, 1997, 2009).

2.1.2. Extreme xerophiles

Dry foods can also be spoiled by extreme xerophiles. These fungi are those that have an absolute requirement for, rather than tolerance of, reduced a_W (Hocking, 2001). Some of the most common fungi in this category are *Bettsia* (= *Chrysosporium pro parte*) species (*B. fastidia, B. alvei,* and *C. xerophilum*), *Xerochrysium* (= *Chryosporium pro parte*) species (*X. dermatitis* and *X. xerophilum*), *Wallemia sebi* and *Xeromyces bisporus* (Pitt and Hocking, 2009; Pitt et al., 2013). *Xeromyces bisporus* is able to grow at a lower a_W (0.61–0.62) than any other known organism (Pitt and Christian, 1968; Hocking and Pitt, 1999; Grant, 2004; Leong et al., 2011, 2015; Williams and Hallsworth, 2009). It produces heat-resistant ascospores and some are able to survive heating at 80 °C for 10 min (Hocking, 2001). Ascospores of *X. bisporus* appear to be capable of surviving the baking process, leading to sporadic spoilage of baked goods which is often not manifested until many months after production. This fungus has never been isolated from soil or decaying vegetation. The most likely sources of *Xeromyces* infections appear likely to be dried fruits, spices and sugary foods with low a_W (Pitt and Hocking, 1997; Leong et al., 2011). *Xeromyces bisporus* can become established in processing plants producing low a_W products providing a low-level inoculum for those products (Hocking and Pitt, 1999). Careful attention to cleaning of all product residues from production lines and equipment before sanitation is essential to eliminate this type of spoilage (Hocking and Pitt, 1999).

Extreme xerophilic fungi will not grow at all in normal (high a_W) media (Pitt and Hocking, 1997, 2009). Extreme xerophilic fungi grow slowly if at all on DG18. The most effective medium suitable for all except the halophilic xerophiles, is malt extract 50% glucose agar (MY50G; 0.89 a_W) (Hocking and Pitt, 1999; Pitt and Hocking, 1997, 2009; Ryu and Wolf-Hall, 2015). Some of these fungi such as *X. bisporus* will not compete against the fast-growing *Aspergillus* (with eurotium-morph) species (*Aspergillus* sect. *Aspergillus*) at a_W above about 0.75 (Hocking and Pitt, 1999). In the presence of such fungi, the most satisfactory isolation medium is malt extract yeast extract 70% glucose fructose agar (MY70GF) which is near 0.75 a_W.

These fungi are usually sensitive to diluents of high a_W and often cannot be isolated by dilution plating (Hocking and Pitt, 1999; Pitt and Hocking, 2009). Dilution plating with low a_W diluents such as 50% glucose and 40% glycerol is possible but less practical because of their high viscosity (Hocking and Pitt, 1999). Direct plating placing small pieces (3–6 per plate) without surface disinfection onto MY50G is recommended. Plates should be incubated upright at 25 °C for 1–3 weeks (Pitt and Hocking, 1997). Plates should be incubated in polyethylene bags to prevent desiccation (Hocking et al., 2006).

Direct sampling by the press plating technique can also be useful (Pitt and Hocking, 2009). Sterile forceps are used to press pieces of the dry food onto a suitable medium such as MY50G (Pitt and Hocking, 1997, 2009; Samson et al., 2004a). The sample is then removed, leaving an impression, and any spores or mycelium transferred will form colonies within a few days or weeks.

If some of the *Aspergillus* (mainly those with eurotium-morph) species that can grow at very low a_W are also found in the dry food sample, they may make the isolation of extreme xerophiles found in the sample more difficult (Hocking and Pitt, 1999; Pitt and Hocking, 1997, 2009). In this case, samples can be plated onto malt extract yeast extract 70% glucose fructose agar (MY70GF; near a_W 0.75). This medium contains equal parts of glucose and fructose to prevent crystallization of the medium at 70% w/w. Even growth of extreme xerophiles on this medium is extremely slow, and plates should be incubated for at least 4 weeks at 25 °C (Pitt and Hocking, 2009). Once growth is apparent, a small portion of the colony can be transferred to MY50G, to allow more rapid growth and sporulation.

2.2. Heat-resistant fungi

Spoilage of thermally processed products by heat-resistant moulds (HRM) is a widespread problem for the food and beverage industries. They are responsible for the spoilage and degradation of heat-processed fruit, fruit juices and purees, and juice-containing beverages as well as shelf-stable teas, sport and energy drinks, flavoured mineral waters, baby fruit gels and coconut water among others. They can grow at low pH and low oxygen partial pressure. These moulds can survive not only the temperatures applied during pasteurization but also the high pressure used during high-pressure processing (HPP) causing numerous incidents of spoilage and significant economic losses. HRM are also able to produce several mycotoxins that pose a hazard to human health. Frąc et al. (2015) has written a comprehensive review on the occurrence of HRM, detection and characterization methods and production of mycotoxins as well as on the inactivation kinetics and control methods.

The ability of HRM to survive these elevated temperatures is due to the formation of highly heat-resistant spores called ascospores (Dijksterhuis, 2007). The thermal treatments given to juices and beverages will not prevent spoilage of these products by HRM (Evelyn and Silva, 2015; Evelyn et al., 2016; Scaramuzza and Berni, 2014; Tranquillini et al., 2017). In general, thermal conditions required to inactivate ascospores of heat-resistant fungi in these products would decrease their sensory quality as well as reduce their nutrient content. Combination of heat treatment and high-pressure processing (high pressure thermal processing, HPTP) or power ultrasound (thermosonication, TS) have also been studied to reduce the treatment temperatures and/or processing times for HRM ascospores (Evelyn and Silva, 2015, 2017; Evelyn et al., 2016). Most of these treatments did not completely inactivate the HRM ascospores.

Species belonging to the genera *Paecilomyces* (with byssochlamys-morph), *Aspergillus* (with neosartorya-morph) and *Talaromyces* have been most frequently isolated from spoiled products (Frąc et al., 2015). Ascospores of the genera *Penicillium* (previously classified as *Eupenicillium*), *Hamigera, Thermoascus, Rasamsonia* and *Monascus* are less commonly occurring. Species in the genera *Hamigera* and *Thermoascus* form ascospores that are highly heat resistant (Hosoya et al., 2014; Scaramuzza and Berni, 2014).

Ascospores of HRM can be found in the ingredients (liquid and dry), packaging and the processing environment. Even though high contamination of the ingredients with HRM ascospores can cause high rates of spoilage in canned fruits, juices and beverages, packaging and processing environment contamination can also contribute to their spoilage. The contamination via packaging and processing will give background spoilage rather than incidental spoilage cases as it is the case with contaminated ingredients.

The life cycle of a HRM is shown in Fig. 2. These moulds have an asexual phase that produce non-heat resistant spores called conidia and a sexual phase that produce the heat-resistant ascospores. These ascospores need a trigger such as heating or high pressure to be activated. This activation takes place during pasteurization and hot-filling of beverages or high-pressure processing (HPP). During activation, the thick outer layer is broken. After activation and if the conditions are favorable, the ascospore can germinate and grow, causing spoilage of the product during storage. Prevention of spoilage of heat-processed products by HRM would require reduction or elimination of the contamination of HRM ascospores from the raw ingredients, packaging and the processing environment (Rico-Munoz, 2017).

In the case of raw materials, empty bottles and environmental samples, the ascospores are in dormant state and must be heat-shocked to be activated and enumerated (Rico-Munoz et al., 2015). Activation of ascospores is the principle of most of the conventional HRM test methods. Because of the low concentration

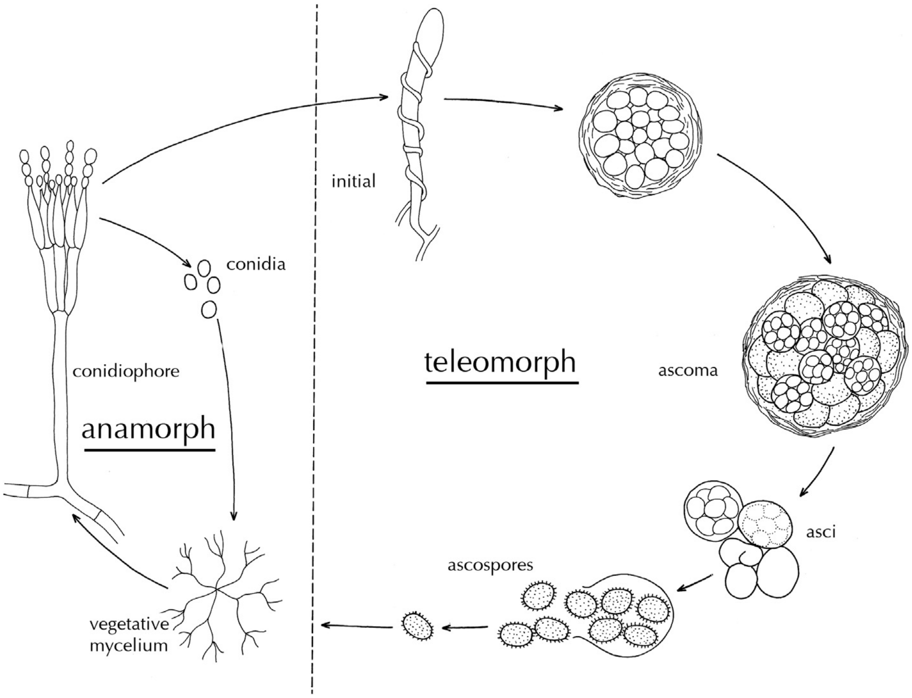

Fig. 2. Life cycle of a HRM — In the life cycle of a heat-resistant mould (HRM) there are two states: (1) an asexual state (left) where not heat-resistant spores such as conidia are formed and (2) a sexual state (right) where the heat-resistant ascospores are formed (courtesy of Rob Samson, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands).

of ascospores in these samples, large samples must be analyzed for their effective detection. Centrifugation or filtration may be used to concentrate the ascospores. Since raw ingredients and environmental samples can be heavily contaminated with bacterial spores, the addition of antibiotics to the plating medium is required to inhibit the bacteria growth.

The latest methodology for heat-resistant mould testing can be found in the 5th edition of the Compendium for the Microbiological Examination of Foods (Rico-Munoz et al., 2015). The methods described in this chapter are cultural methods and can take from 7 to 30 days for the detection of ascospores in the samples. However, the method can detect all of the different heat-resistant moulds present in the sample. The isolated HRM can be identified by molecular methods and should be confirmed by phenotypic characteristics. This method should not be applied to products which have already had a heat treatment step, since this is often sufficient to activate the ascospores. In this case, other isolation methods as the ones described by Pitt and Hocking (2009) or Samson et al. (2010) should be used. The new nomenclature of the HRM must be followed when identifying the isolated cultures (Barbosa et al., 2017; Houbraken and Samson, 2011, 2017; Houbraken et al., 2012; Igarashi et al., 2014; Peterson et al., 2010; Samson et al, 2009, 2014; Visagie et al., 2014; Yilmaz et al., 2014). Frąc et al. (2015) have done an excellent review of molecular methods for the detection of HRM. More research is needed in this field.

2.3. *Preservative-resistant fungi*

Few filamentous fungi are able to spoil products containing preservatives such as sorbic, benzoic, propionic and acetic acids and their salts. They are called preservative-resistant moulds or PRM (Samson et al., 2004b). There are two kinds of PRM: (1) the heat-resistant PRM such as *Paecilomyces variotii, P. niveus* (= *Byssochlamys nivea*) and *Monascus* species (*M. ruber, M. pilosus*) and (2) not heat-resistant such as *Penicillium roqueforti, P. paneum* and *P. carneum* as well as the conidia (non heat-resistant spores) of the first group (i.e. *P. variotii,* a very common mould in the processing and packaging environment of these products). The first group produce heat-resistant ascospores and are often found in ingredients such as corn flour (Rico-Munoz, unpublished) or some of the beverage ingredients (Rico-Munoz, 2017). These ascospores can survive the baking and pasteurization processes. The ascospores in the ingredients can also contaminate the surfaces of the equipment before the oven or pasteurizer or filler. Ascospores can then contaminate the open products and survive the heating step. These species grow better at warmer temperatures and spoilage of preserved products by these moulds usually occur during the warmer months. In the case of the second group, the non heat-resistant group, the contamination happens after the heat step given to these preserved products since they are killed by the baking (Knight and Menlove, 2006) or pasteurization processes (Tournas, 1994). Therefore, for preserved foods such as bread to become mouldy, it must be contaminated either from the air, bakery surfaces, equipment, food handlers or raw ingredients after baking during the cooling, slicing or wrapping operations (Knight and Menlove, 2006). In the case of the *Penicillium* species that can grow under refrigeration such as *P. roqueforti*, spoilage of these products may happen more frequently during the colder months. Fluctuation of temperature in the packaging areas must be avoided since it can result in condensation. The condensation on surfaces (walls, ceilings, overhead piping, etc.) can be conductive for mould growth. Furthermore, moisture condensation inside the package, due to packaging of the products prior to being completely cooled, may accelerate mould growth and spoilage (Saranraj and Geetha, 2012). After the heating step, the contamination of preserved foods by PRM should be avoided.

The simplest and most effective way to screen for PRM is to use a medium containing 0.5% acetic acid such as acetic dichloran yeast extract sucrose agar (ADYS) (Frisvad et al., 1992; Frisvad et al., 1992; Ryu and Wolf-Hall, 2015) or malt extract acetic agar (MAA) (Pitt and Richardson, 1973; Samson et al., 2010). MAA is made by adding glacial (16 N) acetic acid to melted and tempered basal medium to give a final concentration of 0.5% (Pitt and Hocking, 2009). These media cannot be held molten for long periods or re-melted because of their low pH (approximately 3.2). The acetic acid does not need to be sterilized before use. ADYS and MAA are suitable media for monitoring raw materials, process lines and products containing preservatives such as preserved breads (Frisvad et al., 1992), preserved corn tortillas (Rico-Munoz, unpublished) and other preserved foods and beverages. They are also very useful to monitor PRM in air samples from processing areas (Fig. 3). Air samples of at least 1 m^3 air are recommended (Rico-Munoz, unpublished). These media are also effective for testing previously isolated yeasts and moulds for preservative resistance (Pitt and Hocking, 2009). ADYS plates should be counted after seven days of incubation because some conidia germinate more slowly on this acetic acid medium than on other media (Frisvad et al., 1992). This seems to also be the case for MAA plates (Rico-Munoz, unpublished).

2.4. *Anaerobic fungi*

Similar to other strictly aerobic microorganisms, the majority of food spoilage moulds have an absolute requirement of O_2 to produce adenosine triphosphate (ATP) via the oxidative phosphorylation pathway (Bailey-Serres and Chang, 2005; Pitt and Hocking, 2009), although a wide diversity of mould species are able to grow under reduced O_2 partial pressure as low as 1% (Nguyen Van Long and Dantigny, 2016). Some *Penicillium* species and many other common food spoilage fungi are inhibited only slightly when grown in nitrogen atmospheres containing approximately 1.0% oxygen (Hocking, 1990; Baert et al., 2007). The growth of *Mucor ambiguus* on Koji agar and *P. roqueforti* on wheat extract agar was reported at O_2 partial pressure of 0.10% (Yanai and Kojo, 1980) and 0.14% (Magan and Lacey, 1984), respectively. *Paecilomyces variotii* produced normal colonies at 25 °C under 650 mm of vacuum (Pitt, unpublished). Oxygen scavengers have been used to reduce the oxygen concentration to levels below 0.1% (Smith et al., 1986; Rice, 1989). Powers and Berkowitz (1990) were able to prevent mould spoilage of ready-to-eat bread for 13 months while in the absence of scavengers, mould spoilage was noticeable after 14 days.

Most spoilage moulds appear to be sensitive to high levels of carbon dioxide (Pitt and Hocking, 2009). *Paecilomyces niveus* and *Paec. fulvus* (=*Byssochlamys fulva*) were capable of growing at atmospheres containing even 60% carbon dioxide and less than 0.5% oxygen (Taniwaki, 1995). *Aspergillus chevalieri* and *Xeromyces bisporus* did not grow in an atmosphere of 80% CO_2 plus 20% O_2 during incubation for 60 days, or in 20% CO_2 and less than 0.5% O_2 (Taniwaki et al., 2009, 2010). These atmospheres affected the viability of their spores. Neither atmosphere inhibited the growth of *Mucor plumbeus, Fusarium oxysporum, Paec. fulvus, Paec. niveus, P. roqueforti, P. commune* and *Aspergillus flavus*. At less than 80% CO_2 plus 20% O_2, patulin, cyclopiazonic acid and roquefortine C were produced at low levels by *Paec. niveus, P. commune* and *P. roqueforti*, respectively. Aflatoxin was not produced at all by *A. flavus*. *Xeromyces bisporus* was capable of growth in an atmosphere containing only 1% O_2, even in the presence of 70–95% CO_2 but not in an atmosphere containing less than 0.5% O_2 and 20% CO_2 (Hocking and Pitt, 1999). It has been reported that fungal growth is reduced but rarely inhibited at CO_2 partial pressures ranging from 50 to 90% (Nguyen Van Long and Dantigny, 2016). Nguyen Van Long et al.

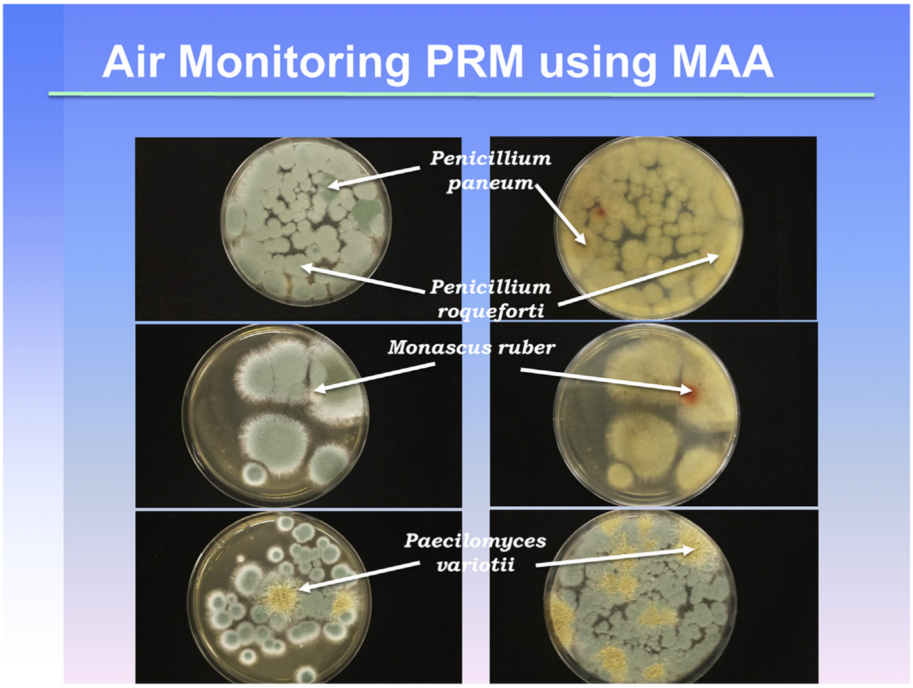

Fig. 3. Air monitoring of preservative-resistant moulds (PRM) using malt extract agar with 0.5% acetic agar (MAA) – colonies of *Penicillium roqueforti*, *P. paneum*, *Monascus ruber* and *Paecilomyces variotii* can be easily detected and isolated.

(2017) confirmed that a modified atmosphere, consisting of O_2 or CO_2 partial pressure higher than 1 and 70%, respectively, was not sufficient to prevent conidial germination of the five fungal species frequently encountered as food spoilers in dairy products or, in the case of *Mucor lanceolatus* and *P. roqueforti*, utilized as cheese ripening cultures.

Mucor plumbeus, *Fusarium oxysporum*, *Paec. fulvus*, and *Paec. niveus* are able to grow under anaerobic conditions (Pitt and Hocking, 2009). Some of those species are able to grow and ferment in bottled liquid products and sometimes cause fermentative spoilage (Pitt and Hocking, 2009). Growth under these conditions may be yeast-like.

As part of the hurdle technology, O_2 and CO_2 hurdle effects should be combined together as well as with other biotic and abiotic factors to prevent fungal spoilage (Nguyen Van Long et al., 2017).

2.5. Psychrophilic and psychrotolerant fungi

There is no consensus on what to call microorganisms that grow at low temperature (Vasavada and Critzer, 2015). Morita (1975) suggested that microorganisms that grow at 0 °C or below which have an optimum temperature of 15 °C and a maximum of 20 °C should be called psychrophiles, while mesophilic microorganisms that grow at 0 °C should be called “psychrotolerant” or “psychrotrophic”.

In general, microorganisms that grow at refrigeration temperatures (0–7 °C) but have temperature optima above 20 °C are called psychrotrophs (Vasavada and Critzer, 2015). Psychrotrophs are defined as microorganisms that produce visible growth at 7 °C ± 1 °C within 7–10 days regardless of their optimum growth temperature (Thomas, 1969). The microorganisms that are most commonly associated with refrigerated foods and cause food spoilage are psychrotrophs and not psychrophiles. Psychrophiles usually die at room temperature or above. Isolation of this group of fungi is difficult and therefore often overlooked (Vasavada and Critzer, 2015).

It is known that psychrotolerant filamentous fungi strains of *Penicillium, Alternaria, Fusarium, Cladosporium* and others grow exceptionally well at low temperatures and are relevant to refrigerated foods (Samson et al., 2004a). The same authors stated that strictly psychrophilic species may not be important in foods but species of *Penicillium* have been found that cannot grow at 25 °C. Ingrain and Mackey (1976) reported that minimum temperatures for fungal growth could occur between −10 °C and −12 °C, but some authors (Leistner et al., 1981) support the view that growth would be possible even at temperatures below −18 °C. According to Pitt and Hocking (2009), low temperatures permissive to fungal growth lie between −7 °C and 0 °C for some species of *Fusarium, Cladosporium, Penicillium* and *Thamnidium*.

Although psychrophilic or psychrotolerant fungi are definitely a problem in foods preserved at low temperatures, the amount of research related to this topic is small (Geiges, 1996; Gill, 2011; Gill and Lowry, 1982). In the literature, research on species of psychrophilic fungi is rare and restricted to temperatures just above zero, although the minimum for growth is generally estimated to be at temperatures slightly below 0 °C (Geiges, 1996). The minimum temperature for growth of fungi may be considerably different depending on the substrate on which the species develops, with lower permissive temperatures when growing in food compared to a conventional culture medium. This is probably because foods which contain proteins, carbohydrates and fats have a higher viscosity providing some protection to the microbial cells during the period in which the product is frozen (Geiges, 1996). Saccomori et al. (2015) reported that *Penicillium polonicum* was able to germinate forming microcolonies (about 1 mm) in 2 of 5 (40%) of the plates of culture media incubated at 0 °C, whereas *P. glabrum* did not germinate in this temperature. Both species formed colonies in the first week when incubated at 5 °C. *P. polonicum* was able to form visible colonies on the surface of nuggets frozen at −5 °C after 17 weeks of incubation. For *P. glabrum*, the minimum temperature at which colony formation was observed was 0 °C, after 9 weeks of incubation. At this same temperature, *P. polonicum* was already forming visible colonies in the 5th week.

No special media has been developed for psychrotolerant filamentous fungi, but incubation temperature is a very important factor to consider and a temperature of 15 °C or 20 °C should be used when working with refrigerated foods (Samson et al., 2004a). The Compendium of Methods for the Microbiological Examination of Foods (CMMEF) 5th edition, chapter 13 (Vasavada and Critzer, 2015), recommends the use of dichloran rose bengal chloramphenicol agar (DRBC) (for foods in general) or dichloran 18% glycerol agar (DG18) for foods with a water activity below 0.95 and incubation of plates at 7 °C ± 1 °C for 10 days or 17 °C ± 1 °C for 16 h, followed by 3 more days at 7 °C ± 1 °C. Some researchers have recommended the use of different media and incubation temperatures and times. Wigmann et al. (2015) recommended the use of potato dextrose agar (PDA) supplemented with chloramphenicol and incubating the plates at 5 °C for 21 days. The medium PDA was selected after comparative tests carried out with DG18 and DRBC, both supplemented with chloramphenicol. PDA had significantly higher recovery of fungi at low temperatures compared to DRBC and DG18, which mostly presented no growth of fungi at all. More research is needed in this area.

3. Molecular detection methodology

Molecular methods for the detection of fungi from spoiled foods are faster than conventional methods. One of their advantages, especially in PCR-based methods, is the specific detection of small amounts of target organisms by amplifying their DNA in a considerably short time frame. The most common used methods are conventional and real-time PCR assays, but next generation DNA sequencing is becoming more common. A compact overview of these methods is given below. Special attention is given to DNA extraction, a crucial step in molecular assays.

3.1. DNA extraction

Extraction of high quality and quantity DNA from fungi can be difficult, even from pure cultures. This becomes even more difficult when the DNA needs to be extracted from a complex matrix, such as food. The DNA extraction protocol therefore has a profound effect on the outcome of a molecular study and needs to be optimized for each food product. One challenge during the extraction is obtaining a sufficient quantity of DNA. Molecular methods usually deal with small amounts of food matrix and therefore also a low copy number will be present in the final DNA extract. Furthermore, fungal growth is often spread heterogeneous through a sample and, similar to culture based techniques, a sufficient sample size should be analyzed (Geisen, 2007). Another challenge is the purity of the DNA. If the DNA is used in a PCR based assay, then compounds in the DNA extract can affect or inhibit the PCR reaction by binding or denaturing the Taq DNA polymerase (Hayat et al., 2012). Commercial DNA extraction kits are available and several house-made DNA extraction methods are described (Grube et al., 2015; Hohnadel et al., 2014; Rodríguez et al., 2012). Studies have shown that different DNA extractions result in differences in the detected microbial community composition. For example, DNA was extracted more efficiently after bead beating of 45 or 450 s (compared to no or a 5 s treatment) (DeSantis et al., 2005). Reproducible DNA extraction of fungi can also be challenging because of the diverse structures they produce. Fungi can produce single celled structures (e.g. conidia in *Penicillium*, *Aspergillus*), multi-celled structures (e.g. *Alternaria*, *Fusarium*) and hyphae. These structures can be thin-walled but can also be thick-walled and/or melanized. Certain species such as *Cladosporium* and *Alternaria* (Dothideomycetes) produce resistant, melanized cell walls and breaking of those cells is difficult, even by bead beating. It is expected that the DNA recovery will be different for each cell type (Summerbell et al., 2011).

3.2. PCR and qPCR methods

Conventional polymerase chain reaction (PCR) and real-time or quantitative PCR (qPCR) assays can be rapid alternatives to traditional culturing and identification methods. In food microbiology, qPCR finds several applications, such as quantification of pathogens, detection of beneficial microbial populations or the study of growth dynamics and metabolic activities for example during cheese ripening (Postollec et al., 2011). qPCR assays developed for detection purposes mostly focus on the important mycotoxin-producing fungi (Atoui et al., 2007; Mayer et al., 2003; Nicolaisen et al., 2009), and the development of assays for spoilage fungi is more limited (Geisen, 2007; Rawsthorne and Phister, 2006).

Methods based on PCR technology have a theoretic detection limit down to 1 to 10 molecules. However, these methods are less sensitive in practice, and sensitivity depends on the quality of the DNA extract, PCR conditions and primers. In a conventional PCR assay, the result of the PCR is visualized at the end of the reaction. With qPCR, the data of amplification is displayed after each cycle. Conventional PCR is mainly used to evaluate the presence of fungi, without information about the copy number in the sample. The copy number can be quantified in a qPCR assay by using an (internal) standard, and the possibility for quantification is an interesting tool if detection alone is not sufficient. Similar to culture-based quantification methodologies, qPCR assays have limitations for quantification. A limitation could be the use of repetitive regions as target for enumeration. For example, ribosomal RNA genes (rDNA) are highly repetitive in fungi, and 40–240 copies per genome might occur (Griffin, 1994; O'Donnell, 1992). When single copy genes are targeted, a 1: 1 ratio between the genome and cell number is expected (Geisen, 2007). However, fungal cells such as hyphae, conidiophores and even unicellular conidia, can be uninucleate or multinucleate (Ishi et al., 2005; van Leeuwen et al., 2013). This can negatively influence the correlation between the estimations of biomass using a culture-based technique and qPCR data (Geisen, 2007). In addition, and mentioned above, the DNA extraction efficiency can influence the quantification by qPCR.

Conventional or real-time PCR based assays detect only what they are designed for and this limits the use of these assays for the detection of the general food mycobiota. Distinguishing dead from living material is technically difficult. cDNA templates, generated via reverse transcription, can be targeted and this can be used to specifically identify metabolically active and viable microorganisms. RT-PCR was developed for the detection of viable yeasts and moulds contaminating yogurts (Bleve et al., 2003) and yeasts in wine (Hierro et al., 2006). Most molecular methods have not been validated. Internal controls can be used to determine the presence of inhibitory substances or failure of a PCR (Hoorfar et al., 2004). False negatives can be obtained when a primer or probe does not bind to variant sequences. On the other hand, primers and probes can also bind to homologous sequences leading to false positives. With the increasing number of genome sequenced strains, validation of these techniques can be partially performed *in silico* but challenging the assays with a large fungal diversity is nevertheless a prerequisite (Summerbell et al., 2011).

After conventional PCR (1st generation) and qPCR (2nd generation), digital PCR (dPCR; 3rd generation) can overcome certain difficulties encountered with qPCR, such as PCR inhibition and the use of an external standard curve. The detection limit of dPCR is lower and can therefore be a promising tool when low copy number need to be detected (Rački et al., 2014).

3.3. *Next generation sequencing*

Next generation sequencing (NGS) is a powerful tool and has the advantage of simultaneous identification of multiple contaminants, and it is even able to discover new and unexpected food spoilage organisms. In a metagenetics approach, amplicons of one of the ITS regions (mainly ITS1) are generated to estimate the mycobiota of food and beverages. The use of the ITS region for characterization of the mycobiota has limitations, as the region has limited resolving power for the identification of foodborne fungi (see below). An approach, in which the total genomic content from a matrix is sequenced including taxonomic relevant genes as well as functional genes, refers to the term metagenomics. In a metagenomics approach, other sequenced genes could be used for identification, resulting in more accurate results. The use of NGS in food sciences will be a great leap forward. As for other methods described above, DNA needs to be extracted from a matrix. Disadvantages are the need of well-equipped and trained laboratories, and the impossibility to discriminate between metabolically live and dead microorganisms (Cardinali et al., 2017; Rodríguez et al., 2015).

4. Identification and typing

Identification of unknown isolates is of importance and a name will unlock a huge amount of additional information. When the species name of the unknown isolate is known, then additional information linked to that name can be found in the literature. Information on the ecology of the species and data on e.g. heat- or preservative resistance, ability to grow at low temperatures and mycotoxin production can be retrieved, and this information can directly affect decision making. Several names of commonly known food associated fungi changed in the last decade due to the changes in the nomenclatural rules, new insights in the classification and the description of new species. More information on the current taxonomy of foodborne fungi can be found in the recent review of Houbraken and Samson (2017). Strain typing – distinguishing between different strains of the same species – is used to get insight in the genetic diversity of spoilage agents (is contamination caused by the same strain) or can be used to trace the source of a contamination. Similar to identification, also typing will attribute to take correct preventative actions. Identification and typing are two overlapping fields and typing often follows identification. However, some typing methods are also (increasingly) used for the recognition of species (e.g. multilocus sequence typing (MLST)). Over the years, many different typing methods are developed, and this manuscript will only focus on amplified fragment length polymorphism (AFLP) typing, multilocus sequence typing (MLST) and short tandem repeat (STR) analysis.

4.1. *Identification*

4.1.1. *Sequence based identification*

In the past, identification of food associated fungi was mainly based on examination of phenotypic characters. Identification based on phenotypic characters can be time consuming and well-trained staff is needed and is therefore more prone to erroneous identifications than a sequence-based identification. In a sequence-based approach, normally one or two genes or loci are being sequenced. The ITS rDNA region was accepted as the official DNA barcode for fungi (Schoch et al., 2012). However, the resolution of this region can be insufficient for species level identification, especially in genera associated with food, such as *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium* (Samson et al., 2004a). No universal secondary barcode is accepted, and which gene should be targeted depends on the genus of your unknown isolate. For example, sequencing a part of the calmodulin gene is recommended in order to reliably identify an unknown *Aspergillus* isolate, while a part of the β-tubulin gene needs to be amplified and sequenced when dealing with a *Penicillium* culture. An overview of recommended loci for identification of most important food associated fungi is given in Table 1. For sterile or unknown isolates, it is recommended to first sequence the ITS region. Public databases such as GenBank are well-stocked with ITS sequences. Comparison of the generated ITS sequence against (public) sequence databases will likely reveal the genus of the unknown isolate, and depending on the genus, the species might also be revealed. In genera where ITS sequencing cannot be used for species recognition, additional sequencing (of a secondary barcode) is needed. It is important to stress that the result of a sequence-based identification heavily depends on the quality of the database. Errors in fungal identifications in GenBank have been found to be as high as 20% (Houbraken and Samson, 2017; Nilsson et al., 2006). More information on identification of foodborne fungi can be found in Houbraken and Samson (2017). In order to prevent (obvious)

Table 1
Overview of important foodborne genera and their recommended genes/loci for identification.

Genus	Gene/locus	Primers	Reference
Alternaria	*GAPDH* and/or ITS	(GPDH: GPD1, fwd) CAACGGCTTCGGTCGCATTG (GPDH: GPD2, rev) GCCAAGCAGTTGGTTGTGC (ITS: V9G, fwd) TTACGTCCCTGCCCTTTGTA (ITS: LS266, rev) GCATTCCCAAACAACTCGACTC	Woudenberg et al., 2013
Aspergillus	*CaM*	(cmd5, fwd) CCGAGTACAAGGARGCCTTC (cmd6, rev) CCGATRGAGGTCATRACGTGG	Samson et al., 2014
Cephalotrichum, *Cladosporium*, *Scopulariopsis*,*Trichoderma*	*TEF1*	(EF1-728F, fwd) CATCGAGAAGTTCGAGAAGG (TEF1-LLErev, rev) AACTTGCAGGCAATGTGG	Bensch et al., 2015; Druzhinina et al., 2005; Kopchinskiy et al., 2005
Fusarium	*RPB2*	(*RPB2*: 5f2, fwd) GGGGWGAYCAGAAGAAGGC (*RPB2*: 7cr, rev) CCCATRGCTTGYTTRCCCAT	M. Sandoval, pers. comm.
Monascus, *Paecilomyces*, *Penicillium*, *Talaromyces*	*BenA*	(Bt2a, fwd) GGTAACCAAATCGGTGCTGCTTTC (Bt2b, rev) ACCCTCAGTGTAGTGACCCTTGGC (T10, fwd) ACGATAGGTTCACCTCCAGAC	Barbosa et al., 2017; Samson et al., 2009; Samson et al., 2014; Visagie et al., 2014; Yilmaz et al., 2014
Rhizopus, *Mucor*, yeasts[a]	ITS and/or LSU[b]	(ITS: V9G, fwd) TTACGTCCCTGCCCTTTGTA (ITS: LS266, rev) GCATTCCCAAACAACTCGACTC (LSU: LR0R, fwd) ACCCGCTGAACTTAAGC (LSU: LR5, rev) TCCTGAGGGAAACTTCG	Dolatabadi et al., 2014; Vu et al., 2016; Walther et al., 2013

Abbreviations: *BenA* = β-tubulin; *CaM* = calmodulin; *GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase; LSU = nuclear ribosomal RNA large subunit; *RPB2* = DNA-directed RNA polymerase second largest subunit; *TEF1* = translation elongation factor 1-alpha.

[a] For the identification of some closely related yeast species, sequencing of an additional marker is needed.

[b] Divergent copies of ITS have been found in Mucorales and certain yeast species. In those cases, sequencing a part of the LSU region is recommended.

misidentifications, it is strongly recommended to use sequence-based techniques in conjunction with morphological techniques.

4.1.2. MALDI-TOF MS

The matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technique can be used to identify bacteria, yeasts and filamentous fungi. MALDI-TOF MS has a short turnaround time, is robust and can be reliable. The technique measures the difference in size of biomolecules (e.g. proteins, peptides, glycoproteins biomarkers, sugars) and organic molecules. MALDI-TOF MS has so far mostly been applied in clinical diagnostics and is mainly used for the identification of bacteria. However, MALDI-TOF MS has also been successfully applied for the routine identification of clinical yeasts (Hendrickx et al., 2011; Kolecka et al., 2013, 2014; Marklein et al., 2009), and can also be used for the identification of foodborne yeasts (Agustini et al., 2014; Pavlovic et al., 2014; Quintilla et al., 2018). MALDI-TOF MS is more challenging for filamentous fungi and this technique is not yet commonly used for the identification of foodborne fungi.

An important step in the identification process using MALDI-TOF MS is the comparison of the generated spectrum against the reference database with mass spectra. The quality and species/strains diversity within the database are essential for a correct identification (Spitaels et al., 2014; Vallejo et al., 2013). Next to the database, the technical variation resulting from the sample preparation procedure is of importance (Usbeck et al., 2013). A large number of publications contain different (1) protocols for protein extraction, (2) matrices for MALDI-TOF MS analysis and (3) growth conditions for fungi. These can affect negatively the spectra quality and consequently fungal identification (Lima and Santos, 2017).

4.2. Strain typing

4.2.1. From multilocus sequence analysis to whole genome sequence typing

Sequencing of one or more loci is applied to identify isolates and when more (normally seven to ten) loci are being sequenced (= multilocus sequence typing, MLST), then this data can be used to identify individual microbial isolates. In medical mycology, this technique has become the number one typing method for microorganisms and this technique is frequently used in epidemiological studies. MLST is accessible, rigorous, reproducible, and informative and can be used for simultaneous identification and strain typing. However, in some genera such as *Aspergillus*, the resolving power of MLST below the species level can be disappointing (Bain et al., 2007). In food mycology, the application of MLST is limited. In this area, MLST is applied for species identification; to a lesser extend for typing strains below the species level (Gillot et al., 2015; Houbraken et al., 2008).

Sequencing of specific loci by Sanger or first-generation sequencing is being replaced by second- and third-generation sequencing methods. Although MLST is simpler, faster, and less expensive than whole genome sequencing, it is more costly and time-consuming than less reliable genotyping methods (e.g. amplified fragment length polymorphisms). Although whole genome sequence typing (WGST) is becoming more accessible, especially for organisms with small genomes, such as bacteria and viruses, it is not yet practical for genotyping numerous fungal isolates. Nevertheless, with the fast development in this area and in bioinformatics, it can be expected that WGST will become the standard in the coming years. Next generation sequencing approaches are nevertheless used to develop new MLST methods. For example, Next Generation Multilocus Sequence Typing (NGMLST) uses the next-generation sequencing technology to rapidly and economically MLST genotype 96 or more isolates in a single assay (Chen et al., 2015).

4.2.2. AFLP

Amplified fragment length polymorphism (AFLP) is a genotyping technique based on PCR amplification of specific restriction fragments from a particular genome and development of AFLP fingerprinting requires no prior sequence information. The discriminatory power of AFLP equals that of the STR panels, however, its long-term stability and reproducibility is challenging (de Valk et al., 2007a; Klaassen and Osherov, 2007). This technique outperforms Random Amplified Polymorphic DNA (RAPD), which is based on the amplification of random genomic DNA fragments by arbitrarily selected primers (Meudt and Clarke, 2007).

In the food production environment, the AFLP method was applied for tracing the contamination source and to pinpoint critical points in a factory. For example, Kure et al. (2003) used AFLP for characterization of *Penicillium commune* and *P. palitans* isolates on, as well as below, species level. Combined M13 fingerprints with AFLP data could relate strains isolated from cheese to specific points in the production plants. Similarly, Lund et al. (2003) compared AFLP with RAPD fingerprints of *P. commune* and showed that discriminatory power of AFLP was higher compared to RAPD. Also, phenotyping was applied and these results match with those of the AFLP analysis. Besides revealing critical points in the production process, this study also showed that several *P. commune* isolates remained present in the environment for more than 7 years. AFLP is also extensively used to study the genetic difference within a species or between closely related species. For example, Montiel et al. (2003) used AFLP to study the isolates belonging to the *Aspergillus* section *Flavi* complex. AFLP was able to separate *A. sojae* from *A. parasiticus*, but it was not able to consistently distinguish between *A. oryzae* and *A. flavus*. The distinction between *A. parasiticus* and its domesticated form *A. sojae* is interesting, because those species are conspecific and named differently only for applied reasons.

4.2.3. Microsatellite analysis

Microsatellites (or short tandem repeats, STR's) are ubiquitously present in the genomes of many fungi. Microsatellite polymorphisms are based on the number of repeats of short DNA fragments, mostly outside coding regions, and are thus expected to be more polymorphic and more rapidly evolving than MLST markers. MLST can be used for species identification, and microsatellite analysis is not commonly used for that purpose. The use of microsatellites offers a number of advantages over many other fingerprinting techniques, including the ease of amplification, multiplex options, extremely high discriminatory power and highly portable and exchangeable typing result (Klaassen and Osherov, 2007). Similar to the other typing techniques mentioned above, this method is mainly applied in the medical mycology, and microsatellites are developed for e.g. *A. fumigatus*, *A. terreus*, and *A. flavus* (de Valk et al., 2007b; Lackner et al., 2016; Rudramurthy et al., 2011). On the other hand, microsatellites are also used in relation to food (production) and studies on the genetic diversity of foodborne fungi, for example in *Aspergillus flavus* (Tran-Dinh and Carter, 2000), *Aspergillus niger* (Esteban et al., 2008), *Penicillium chrysogenum* (Henk et al., 2011), *Penicillium roqueforti* (Gillot et al., 2015) and *Penicillium commune* (Giraud et al., 2010).

5. Conclusions

Mould spoilage prevention requires knowledge of the associated mycobiota of that food or beverage. Mould spoilage is becoming widespread and fungi have an excellent capacity to adapt to any hurdle which is currently used for the prevention of spoilage. It is important to tackle any mould problem with the specific mycological methods including the appropriate media. While classic mycological detection methods can detect a broad range of fungi using well validated protocols, they are time consuming and results can take days or even weeks. New molecular detection methods are faster but require good DNA isolation techniques, expensive equipment and may detect viable and non-viable fungi that probably will not spoil a specific product. Although there is no complete and easy method for the detection of fungi in food it is important to be aware of the limitation of the methodology.

Declaration of interest

None.

Conflicts of interest

None.

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