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Heat-Resistant Moulds in Fruits and Fruit-Containing Products

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Abstract: Heat-Resistant Moulds (HRM) are defined as microorganisms that can survive thermal processes applied to foods due to their ascospores and can develop during storage. The most significant feature that makes these moulds important in the food industry is their ability to reactivate during thermal processes like pasteurisation, thermisation, and other processes involving low oxygen and high pressure, as well as being present in the sexual phase. The most common HRMs include *Byssochlamys* spp., *Neosartorya* (*Aspergillus*) spp., *Talaromyces* spp., *Eupenicillium* spp., *Hamigera* spp., and *Thermoascus* spp. The primary source of HRMs is soil and fruits that come into contact with the soil. Especially strawberries, raspberries, grapes, apples, blueberries, and blackberries are among the most frequently studied products in the literature. In addition, HRMs have been isolated from and identified in various processed products such as fruit juices, baby food, puree-jams, marmalades, dairy products, and olives using different methods. Enzymes and mycotoxins synthesized by HRMs not only spoil food, causing economic losses, but also pose a hidden risk to public health. Therefore, the quality of raw materials, storage conditions, food hygiene practices, proper temperature pasteurisation, and the use of preservatives gain importance. This review aims to provide an additional perspective to the literature on the nomenclature of HRMs, their heat resistance, commonly found species in foods, metabolites produced by HRMs, and detection and prevention methods.

Keywords: HRM, Ascospore, Food, Fruit, Heat resistance, Mould

Meyve ve Meyve İçeren Ürünlerde Isıya Dirençli Küfler

Öz Isıya Dirençli Küfler (HRM), askosporları sayesinde gıdalara uygulanan ısısal işlemlere dayanabilen ve depolama sırasında gelişebilen mikroorganizmalar olarak tanımlanmaktadır. Bu küfleri gıda endüstrisinde önemli kılan en önemli özellik, pastörizasyon, termizasyon gibi ısıl işlemler ile düşük oksijen ve yüksek basınç içeren diğer işlemler sırasında yeniden aktif hale gelmeleri ve seksüel fazda bulunmalarıdır. En yaygın HRM'ler arasında *Byssochlamys* spp., *Neosartorya* (*Aspergillus*) spp., *Talaromyces* spp., *Eupenicillium* spp., *Hamigera* spp. ve *Thermoascus* spp. bulunmaktadır. HRM'lerin ana kaynağı toprak ve toprakla temas eden meyvelerdir. Özellikle çilek, ahududu, üzüm, elma, yaban mersini ve böğürtlen literatürde en sık incelenen ürünler arasındadır. Ayrıca HRM'ler, farklı yöntemler kullanılarak meyve suları, bebek maması, püre-reçel, marmelat, süt ürünleri ve zeytin gibi çeşitli işlenmiş ürünlerden izole edilmiş ve tanımlanmıştır. HRM'ler tarafından sentezlenen enzimler ve mikotoksinler, yalnızca gıdayı bozmakla kalmayıp ekonomik kayıplara da neden olmakta ve ayrıca halk sağlığı için gizli bir risk oluşturmaktadır. Bu nedenle ham maddelerin kalitesi, depolama koşulları, gıda hijyeni uygulamaları, uygun sıcaklıkta pastörizasyon ve koruyucu madde kullanımı önem kazanmaktadır. Bu derlemenin amacı, HRM'lerin isimlendirilmesi, ısı direnci, gıdalarda yaygın olarak bulunan türler, HRM'ler tarafından üretilen metabolitler ve tespit ve önleme yöntemleri konularında literatüre ek bir bakış açısı sağlamaktır.

Anahtar kelimeler: HRM, Askospor, Gıda, Meyve, Isıl direnç, Küf

Introduction

Heat-resistant Moulds (HRM) can be defined as microorganisms that cannot be inactivated by thermal processes in foods and have the potential to cause spoilage and toxin formation (Maneeboon et al., 2023). The concept of heat-resistant moulds first emerged as a problem in canned strawberries in England in 1934 (Oliver and Rendle, 1934). The heat resistance mechanism of HRMs is reported to arise from the sexual phase of the spores in their structure (Doyle and Sperber, 2009; Frac et al., 2015). Various sources state that HRMs develop resistance through environmental stimuli such as high temperature and pressure, maintaining their activity and causing various spoilages after thermal processing (Ulusoy et al., 2022; Houbraken, 2006; Ishara and Gunasena, 2021; Rico-Munoz et al., 2019).

The primary source of HRM ascospores is soil and products grown in soil, particularly fruits. Studies to date have focused on fruits grown in soil, especially strawberries, apples, pineapples, grapes, tomatoes, blueberries, and various products derived from them such as fruit juices, baby food, sauces, and purees (Rico et al., 2015; Salomão, 2018; Rico-Munoz et al., 2019; Ayva et al., 2019; Kim and Silva 2016; Kelfkens, 2024). In addition, studies have also been conducted on dairy products such as fruit yoghurt, kefir, and ice cream (Aydın et al., 2005; Engel and Teuber, 1991).

Among the most common HRMs causing spoilage in foods are *Byssochlamys* Westling spp. (teleomorph of *Peacilomyces*)*, Neosartorya* Wehmer spp. (teleomorph of *Aspergillus*)*, Talaromyces* Benj.spp.*,* and *Eupenicillium* Ludw (Teleomorph of *Penicillium*) spp. (Kelfkens, 2024). Additionally, *Hamigera* Stolk & Samson *Eurotium* Link, *Monascus* Tiegh and *Rasamsonia* Houbraken & Frisvad spp. are HRMs less frequently isolated from foods (Rico-Munoz et al., 2015; Rico-Munoz and dos Santos, 2019). These moulds are reported to survive at temperatures higher than 75℃ for 30 minutes. Notably, *Paecilomyces niveus* Stolk & Samson (*Byssochlamys*) and *Aspergillus fischeri* Wehmer (*Neosartorya*) have been reported to remain active at this temperature (Silva, 2015; Kim and Silva 2016). *Aspergillus thermomutatus* Peterson, *Thermoascus crustaceus* (Apinis & Chesters) Stolk, and Samson, *Talaromyces macrosporus* (Stolk & Samson) Frisvad, Samson & Stolk have been identified as more heat-resistant in recent years, while *Eurotium* Link species have been defined as resistant to lower temperatures (Dijksterhuis, 2019).

HRMs cause various spoilages by synthesizing enzymes and mycotoxins in foods, which can also lead to serious health problems (Salomão, 2018). Therefore, HRM detection and prevention are necessary. Various methods have been used for HRM detection (Kotzekidou, 2014). Studies on HRMs are quite limited in literature. This study examines HRMs under the headings of Nomenclature for HRM, HRM Ascospores and Their Thermal Resistance, Most Common HRMs Isolated from Foods, and Detection of HRM.

Taxonomy and Nomenclature for HRM

Moulds can exhibit different morphs (reproductive forms) within a single organism. Previously, it was possible to name the same mould with different names due to its different forms (anamorph, teleomorph, synanamorph) and structures. However, the current nomenclature for HRMs follows the "One mould - one name" rule, considering only asexual forms (Rico-Munoz et al., 2015). This naming system was adopted to eliminate the confusion in the binary naming of HRMs, which previously considered their sexual (reproductive) forms. This complexity especially affects the genus *Talaromyces*, which is classified as asexual in two different genera, *Penicillium* and *Paecilomyces* (Kelfkens, 2024). Additionally, in some sources, HRMs are named with both asexual and sexual forms by appending the sexual form to the asexual name (Rico-Munoz et al., 2019; Rico-Munoz and dos Santos, 2019; Kim and Silva., 2016; Stefanello et al., 2020). The current nomenclature was given in Table 1.

HRM Ascospores and Their Thermal Resistance

Filamentous fungi reproduce in two ways, asexually and sexually. While asexual reproduction involves mitotic processes, sexual reproduction consists of meiotic stages that form spores. It is known that more than 25% of all mould species reproduce only asexually and do not have any teleomorph forms (Maj et al., 2023). HRMs, which belong to the class *Ascomycotina*, are microorganisms that possess both an asexual phase, producing non-heat-resistant conidia spores, and a sexual phase, producing heat-resistant spores during their life cycle (Pitt and Hocking, 2009). Heat-resistant ascospores generally form from asci in groups of eight. The genus *Byssochlamys*, characterised by its ascospores, stands out in this regard. Some genera, however, are known for their structures called ascoma or ascomata, also called "fruiting bodies." For example, *Talaromyces* gymnothecium, *Neosartorya*, and *Eupenicillium* are known for their cleistothecium, which provides resistance to heat (Pitt and Hocking, 2009; Pitt and Hocking, 1984; Samson et al, 2007, 2014; Yılmaz et al 2014). While gymnothecium exhibits a completely enclosed, spherical or pear-shaped structure, cleistothecium consists of tufts formed by spiralled and interwoven spiny hyphae (Maj et al., 2023; Pitt and Hocking, 1984).

Table 1. New nomenclature for HRM (Dijksterhuis, 2019; Kelfkens, 2024; Rico-Munoz and dos Santos, 2019; Rico-Munoz et al., 2019; Lane, 2019; Tournas, 1994).

Old Nomenclature	New Nomenclature
Byssochlamys fulva Olliver & G. Sm	*Paecilomyces fulvus* Stolk & Samson
Byssochlamys nivea Westling	*Paecilomyces niveus* Stolk & Samson
Byssochlamys spectabilis (Udagawa & Shoji Suzuki) Houbraken & Samson	*Paecilomyces variotii* Bainier
Neosartorya fumigata O'Gorman, H.T. Fuller & P.S. Dyer	*Aspergillus fumigatus* Fresen.
Neosartorya hiratsukae Udagawa, Tsub. &. Horie	*Aspergillus hiratsukae* Udagawa, Tsub. & . Horie
Neosartorya glabra (Fennell & Raper) Kozak*.*	*Aspergillus neoglaber* Kozak. 1989*,*
Neosartorya fischeri i(Wehmer) Malloch & Cain	*Aspergillus fischeri* Wehmer
Neosartorya pseudofischeri Peterson	*Aspergillus thermomutatus (Paden) Peterson*
Penicillium dangeardii Pitt	*Talaromyces flavus (Klöcker) Stolk & Samson*
Eurotium herbariorum (Wigg.) Link ex Nees,	*Aspergillus glaucus* Link
*Eupenicillium javanicum (*Beyma) Stolk & Scott	*Penicillium javanicum* Beyma
Penicillium macrosporum Frisvad, Filt., Samson & Stolk	*Talaromyces macrosporus* (Stolk & Samson) Frisvad, Samson & Stolk
Backusia terricola Thirum, Whitehead & Mathur	*Monascus ruber* Tiegh
Penicillium avellaneum Thom & Turesson	*Hamigera avellanea* Stolk & Samson
Dactylomyces crustaceus Apinis & Chesters	*Thermoascus crustaceus (Apinis & Chesters) Stolk*

The thermal resistance of HRMs is suggested to arise from the ascospores produced in the sexual phase (Doyle and Sperber, 2009; Frac et al., 2015). Ascospores generally exhibit higher thermal resistance than the conidia seen in the asexual phase and can withstand pasteurisation processes applied to fruit products and beverages (Dijksterhuis and Teunissen, 2004; Ray and Bhunia, 2014). Additionally, thermal treatment can stimulate ascospores, triggering their germination (Kelfkens, 2024).

Sesli et al. (2020) gave Turkish names to the fungi isolated from various sources in Turkey. Among these, there are some heat-resistant moulds.

The ascospores of *Neosartorya spp* (Turkish name: Gavur asper), *Byssochlamys spp* (Turkish name: pembecil), and *Talaromyces spp* (Turkish name: süpürgen) are the most commonly known and isolated HRMs to date and are responsible for spoilage in processed foods (Ishara and Gunasena, 2021; Sesli et al., 2020). HRMs pose a significant risk to the industry as they can survive and grow not only during thermal processes such as pasteurisation and thermization but also under conditions of high pressure, low oxygen, and low humidity (Kim and Silva., 2016; Rico-Munoz and Dos Santos, 2019; Dos Santos et al., 2019; Dos Santos and Samapundo et al., 2020 Dijksterhuis, 2019). In one study, *Byssochlamys nivea (*Turkish name:Pembecil), *Neosartorya fischeri (*Turkish name:Gavur asper), *Talaromyces avellaneus (*Turkish name:Elpudra), and *Penicillium expansum (*Turkish name: Geniş penisilyum) were able to survive at 75°C for 15 minutes in strawberry puree, pineapple nectar, grape juice, and apple juice under pressures ranging from 350 to 700 MPa (Megapascal) (Silva, 2015; Silva, 2017; Silva, 2020). Another study conducted on high-acid pasteurised fruit products (strawberry puree, apple puree, orange juice) reported that the most isolated HRM product was strawberry puree. After pasteurisation, 34.5% of the fruit products were found to be contaminated with HRMs, with the isolated species being *N. fumigate (*Turkish name:Kıran asper) (39.7%), *N. fischeri* (27%), and *B. nivea* (7.9%) (Dos Santos et al., 2019). It was found that *Thermoascus spp***.** could maintain activity at 90°C, while *Byssochlamys spp***.** lost its activity (Yaguchi, 2023). *Aspergillus fumigatus* ascospores were able to survive at 80-85-90°C for 30 minutes and were inactivated at 95°C after 15 minutes. In contrast, *Paecilomyces variotii (*Turkish name:Gün Küfü*)*, which is less resistant than *Aspergillus fumigatus*, was able to survive at 90°C for 10 minutes (Arıcı, 2006). HRMs, such as *Neosartorya spp***.** and *Byssochlamys spp***.**, have also been reported to grow in strawberry, orange juice, and apple products in low-oxygen headspace conditions (0.03%-0.05%) (Dos Santos et al., 2019). Table 2 shows the thermal resistance of certain species under different environmental and temperature conditions.

Most Isolated from Foods (Common) HRMs

The primary sources of HRMs include areas such as soil, vineyards, and orchards. Since the fruits grown in these areas can naturally be contaminated with HRM spores, they are considered high-risk products (Aydın et al., 2005). Species such as *Byssochlamys nivea*, *B. fulva*, *Neosartorya fischeri*, *Talaromyces avellaneus*, *T.flavus* (Turkish name: sarı süpürgen), *T.macrosporus* (Turkish name: Koca süpürgen) *Eupenicillium javanicum (*Turkish name: Topaç penisilyum), *Penicillium expansum* are defined as causing spoilage in fruits through their spores (Silva, 2020). Foods from which HRMs are commonly isolated include soil-grown fruits, fruit juices, baby food, wine, milk and dairy products, margarine, tomato paste, and baked goods.(Kotzekidou, 1997; Ulusoy et al., 2022; Pitt and Hocking, 1997; Kelfkens, 2024; Dos Santos et al., 2019). Other food products and supplements from which HRM is isolated include sweeteners (granulated, liquid, sugar alcohols), pectin, juice purees, juice concentrates, tea leaves, roots, and vitamin powders. (Arıcı, 2006; Ishara and Gunasena, 2021; Aydın et al., 2014; Kelfkens, 2024; Pahalagedara et al., 2024).

In a study conducted on cherry, peach and raspberry samples, *Talaromyces flavus* and *Neosartorya fischeri* were identified as species with extra heat resistance (Beuchat, 1986). In another study involving frozen strawberries, blueberries, lemon, black carrot extract, and red grape extract, *Talaromyces* spp**.** and *Eurotium* species were the most frequently detected, while no HRMs were found in grapes and carrots (Tranquillini et al., 2017).

A recent study reported that 90.9% of pasteurized strawberries were contaminated with HRMs (Pahalagedara et al., 2024). HRMs have also been isolated from various fruit processing lines and environments.

Table 2. HRM thermal resistances at different media and temperatures

HRM	Media	Heat Resistance	References
Byssochlamys nivea	Grape juice Apple juice Pineapple nectar Strawberry puree	60 min for 88 °C 99°C. survived in juice D= 0.9-55.2 min for 95-104°C D=1.8-13.7 min for 85-90°C	Ulusoy et al. (2022) Dos Santos et al. (2019) Ferreira et al. (2009) Silva (2015)
Byssochlamys fulva	Tomato juice Passion fruit nectar	D90°C =8.1 min D=0.6-27 min for 98-107°C	Rico-Munoz et al. (2015) Ferreira et al. (2011)
Eupenicillium lapidosum	Blueberry Juice	9 min. for 93.3 °C	Ulusoy et al. (2022)
Neosartorya fischeri	Apple juice Fruit-based fiilings Mango drink	3.3 log reductions after 10 min for HPP and 75°C 1.4 min. for 87.8 °C D85°C=56.25 min	Kim and Silva (2016) Souza et al. (2017) Rajashekhara et al. (1996)
Talaromyces trachyspermus	Blueberry and grape juice	2.33-2.85 min for 82°C	Tranquillini et al. (2017)
Talaromyces macrosporus (T. fulvus)	Apple juice	D90.6°C =2.2 min	Rico-Munoz et al. (2015) Dos Santos et al. (2019)
Talaromyces.bacillisporus	Blueberry and grape juice	1.03-1.56 min for 91°C	Tranquillini et al. (2017)
Thermoascus crustaceus	Blueberry and grape juice Grape juice Apple juice	1.11-2.53 min for 95 °C 60 min. survival for 88°C D=1.8-24.4 for 90-95°C	Scaramuzza and Berni (2014) Rico-Munoz et al. (2015)

The most isolated species from beverage processing lines were found to be *Paecilomyces variotii, Aspergillus hiratsukae*, and *Aspergillus fischeri*, in that order (Rico-Munoz and dos Santos, 2019). Species such as *B. nivea*, *B. fulva*, *B. spectabilis*, *N. fischeri*, *T. avellaneus*, *T. macrosporus (*Turkish name: Dikenli yumurta) and *Eupenicillium* spp. are the most common HRMs responsible for spoilage in fruits (Silva, 2020). Table 3 (below) lists the various HRM genera identified over the years in different areas. *Hamigera* (Turkish name: Elpudra*)*, *Thermoascus* (Turkish name: Dikenli yumurta, and *Monascus (*Turkish name: Topuz küf) are less commonly isolated genera.

Limited studies have been conducted on heat-resistant moulds in milk and dairy products, and various species have been identified. It has been reported that while pasteurisation affects ascospores in milk, homogenization and centrifugation do not have any effect (Aydın et al., 2005). *Monascus ruber* was detected at a level of 2.5 CFU/L in raw milk (Engel, 1991). Storing cream cheeses at temperatures above 12°C can encourage the formation of HRM moulds (Pitt and Hocking, 1997). In a study conducted on cheeses (white cheese, tulum cheese, and herb cheese), *T. macrosporus* was not detected, but *B. nivea* was identified (Aydın et al., 2005). As a result, milk and dairy products can be considered a risky product group for HRMs. The addition of fruit-based ingredients to dairy products may further increase the risk of contamination and spoilage. The most commonly isolated species from food include *Byssochlamys* spp., *Talaromyces* spp., *Neosartorya* spp., and *Eupenicillium* (Houbraken et al., 2006; Pitt and Hocking, 2009 Samson et al, 2007, 2014).

Byssochlamys species are abundant in soil and are considered significant moulds in fruit products (Silva, 2015). *Byssochlamys* produces spores in clusters of eight within an ascus. The most commonly cited species in the literature are *B. fulva* and *B. nivea*, while studies on *B. spectabilis* are limited (Kotzekidou, 2014). It has been reported to survive at 90°C for 25 minutes or longer (Tournas, 1994; Kotzekidou, 2014).It can maintain activity in a wide pH range (3-8) and under low oxygen conditions, as demonstrated in pineapple nectar at 103°C for 7 minutes (Ferreira et al., 2011). Apart from pasteurized fruit products, *Byssochlamys* has been detected in various products like cucumber brine, milk, dairy products, and wine samples (Pitt and Hocking, 1997; Aydın et al., 2005; Eziashi et al., 2010). Areas of isolation include strawberry fields, vineyards, rivers, and soils (Piecková et al., 2020). *Byssochlamys* is important to the food industry due to its ascospores high heat resistance (Ulusoy et al., 2022; Dos Santos et al., 2019; Silva, 2015; Rico-Munoz et al., 2015; Ferreira et al., 2011). Its thick cell walls and cytoplasmic membrane protect the ascospores from heat. Low pH, the presence of organic acids and preservatives like SO_2, and high concentrations of sugar and salt can also enhance the ascospores' heat resistance (Kotzekidou, 2014). Due to their ability to produce CO_2 under low oxygen tension (less than 0.5%) in packaging, thermal treatment below 90°C for 10 minutes can allow the germination and growth of ascospores. Therefore, combined technologies such as TS (Thermasonication), HPP (High-Pressure Processing), MAP (Modified Atmosphere Packaging), low-temperature storage, and preservatives are employed (Dos Santos and Samapundo et al., 2020; Silva, 2017; 2015, Nema et al., 2022; Buerman, 2020). Like other HRMs, *Neosartorya* spp. belongs to the Ascomycetes class and Aspergillaceae family (Maj et al., 2023). It is characterised by the cleistothecium structure surrounding the ascospores, which provides heat resistance. The most well-known species are *N. fischeri* and *N. pseudofischeri* (Rydholm et al., 2006). Other commonly isolated species from fruit and soil include *N. spinosa*, *N. glabra*, *N. assulata*, *N. quadricincta*, *N. hiratsukae*, and *N. laciniosa* (Maj et al., 2023). The heat resistance of Neosartorya ascospores has been noted with D-values of D87°C = 3.3-15.4 minutes, D90°C = 1.3-4.3 minutes, and D95°C = 0.3-0.6 minutes in strawberry samples (Berni et al., 2017).

Some sources suggest that *N. fischeri* ascospores are comparable to bacterial spores in terms of heat resistance and are more resistant than *B. fulva*, the most heat-resistant mould (Kim and Silva., 2016; Pitt and Hocking, 1997). Studies have shown that the heat resistance of ascospores increases with age (Slongo et al., 2006; Tournas and Traxler, 1994). There are various studies on this genus, such as a study where a *Neosartorya spp.* was identified as a potential biofertilizer for promoting the growth of Chinese cabbage (Hamayun, 2011). Another study demonstrated antimicrobial activity against bacteria like *Bacillus subtilis* and *Staphylococcus aureus* (Shan et al., 2012). Additionally, after heat treatment at 93°C for 1-8 minutes, proteins produced by *Neosartorya pseudofischeri*, isolated from blueberries, doubled, indicating that heat accelerates their metabolic reactions (Chen et al., 2022).To combat HRMs like *Neosartorya*, strategies involving natural antifungal agents such as microbial-based solutions, plant extracts, and essential oils might be the best approach for preventing food spoilage (Maj et al., 2023). For *Neosartorya* spp. isolated from pasteurized strawberry puree, Quantitative Microbial Spoilage Risk Assessment (QMSRA) can help estimate the potential spoilage of products (Dos Santos and Membré et al., 2020). It was also noted that low water activity (0.87) and oxygen combination (0.15%) are effective against this genus, and small differences in water activity can inhibit *A. fisherianus* (Dos Santos and Samapundo et al., 2020).

Talaromyces spp. is associated with various moulds such as *Penicillium*, *Paecilomyces*, and *Geosmithia*, and its spore structure is an ascocarp called gymnothecium. It is commonly isolated from heat-treated foods and fruit products (Pahalagedara et al., 2024). *T. flavus* (formerly *Penicillium dengaardii*) (*Turkish name: Hoş süpürgen)* is the most frequently isolated species from pasteurized fruit juices (Salomão, 2018). Another species, *T. bacillisporus* (formerly *Geosmithia*), has been detected in processed orange juices (Santos et al., 2018), while *T. trachyspermus* has been isolated from frozen pineapple juices (Enigl et al., 1993). The species *T. flavus* has been analyzed in blueberries, peaches, cherries, raspberries, and strawberries, with D-values at 91°C being 5.4 - 4.2 - 4.9 - 3.4 and 11. 7 minutes respectively (Pahalagedara et al., 2024).

Studies also highlight the role of *Talaromyces* spp. in producing enzymes and mycotoxins with significant biological activity (Zhai et al., 2016; Li et al., 2022; Lan and Wu, 2020). For instance, *Talaromyces marneffei* has been linked to HIV (Human Immunodeficiency) and AIDS (Acquired Immune Deficiency Syndrome) (Sun et al., 2020), while other species have shown anticarcinogenic, antibacterial, and antifungal activities (Kumari et al., 2018; Nicoletti et al., 2018).

Eupenicillium is characterised by cleistothecium ascospores with smooth walls. Although it is not as prevalent in food as *Neosartorya*, *Talaromyces*, *Byssochlamys*, studies on its occurrence in foods are limited (Pitt and Hocking, 2009; Pahalagedara et al., 2024).

The most commonly isolated species from food include *E. brefeldianum*, *E. cinnamopurpureum*, *E. hirayamae*, and *E. javanicum* (Salomão, 2018).

Table 3. Some studies on HRMs from past to present

HRM	Foods	References
Byssochlamys spp.	Canned strawberry	Oliver and Rendle (1934)
Monascus spp., Byssochlamys spp	Raw milk and silage	Frevel et al. (1985)
Talaromyces flavus	Pineapple juice, apple juice, grape juice	Beuchat (1988)
Monascus ruber	Bakery products	Spicher and Isfort (1988)
Monascus ruber	Raw milk	Engel (1991)
Byssochlamys fulva, Byssochlamys nivea, Neosartorya fischeri	Canned Tomato paste	Kotzekidou (1997)
Byssochlamys nivea, T. avellaneus, N. fischeri, Eupenicillium brefeldianum	Cream cheese	Pitt and Hocking (1997)
Neosartorya fischeri *Talaromyces flavus* *Byssochlamys nivea*	Mango concentrate Grapefruit juice Apricot Juice	Heperkan and Vasavada (2003) Samson et al. (2002)
Talaromyces avellaneus	Apple juice	Voldrich et al. (2004)
Talaromyces macrosporus and *Byssochlamys nivea*	Cheese, fruit yoghurt, ice cream, fruit juice	Aydın et al (2005)
Aspergillus fumigatus, Paecilomyces variotii	Margarine	Arıcı (2006)
Byssochlamys nivea	Strawberry pure	Silva (2015)
Neosartorya fischeri	Apple juice	Kim and Silva (2016)
Talaromyces trachyspermus, Talaromyces bacillisporus *Byssochlamys, Eurotium, Neosartorya, Penicillium,Talaromyces*	Blueberry and grape juice Frozen strawberries, Frozen blueberries, Frozen lemon cells	Tranquillini et al. (2017)
Neosartorya fumigata, Neosartorya fischeri Byssochlamys Nivea	Strawberry pure,orange juice, apple püre (pasteurized products)	Dos Santos et al. (2018)
Paecilomyces niveus	Apple	Biango-Daniels and Hodge (2018)
Aspergillus, Bysocchlamys, Talaromyces ,Penicilium	Soil (agricultural land)	Ayva et al. (2019)
Talaromyces macrosporus and *Aspergillus fischeri*	Strawberry pure	Timmermans et al. (2020)
Aspergillus fischeri, Paecilomyces niveus	Apple juice	Buerman et al. (2021)
Thermoascus auranthiacum	Grape Juice (88 °C for 60 min, survival)	Ulusoy et al. (2022)
Aspergillus, Penicillium, Hamigera, Talaromyces, Paecilomyces	Pineapples and sugarcane	Maneboon et al. (2023)
Byssochlamys fulva	Carbonated beverages	Saubade et al. (2024)
HRM Ascospore	Fruit based Baby Food Puree	Kelfkens (2024)

Various environmental isolates of *Eupenicillium* have been found in pastures, rivers, saltwater, and soils (Visagie et al., 2014; Panagou et al., 2010). Other findings suggest that increasing the temperature by 10°C significantly increases *Penicillium brefeldianum* spore activity (Spuy et al., 1975). A study on *E. javanicum* noted that higher Brix° values increased D-values (Muria et al., 2020). *Penicillium expansum*, *P. buchwaldii*, and *P. bialowiezense* have been isolated from fruit juices and smoothies processed with pulsed electric field (PEF). It has been reported that their spores can be inactivated by high-pressure processing (HPP), cold atmospheric plasma, UV (Ultraviolet) and chemical disinfectants such as chlorine dioxide and hypochlorite as preventive approaches (Groot et al., 2019).

Detection of Heat-Resistant Moulds (HRM)

The identification of heat-resistant moulds typically requires the isolation of HRM from samples and their conventional identification. The basic stages of traditional identification are homogenization, heating-cooling (pasteurisation), incubation, and isolation (Rico-Munoz,et al., 2015; Beuchat and Pitt, 2001). Molecular identification involves DNA isolation, PCR, sequencing analysis, and the use of various chemicals and processes (Maneboon et al., 2023; Peterson et al., 2010; Dos Santos et al., 2018). Identification and determination of their heat resistance can be performed through steps such as preparing ascospores, determining viable ascospores, and identifying heat-resistant ascospores.

The fundamental method of HRM detection relies on the inactivation of ascospores and the heat treatment applied (Ulusoy et al., 2022). The plating method and direct incubation method are the two primary methods used in the literature for traditional HRM detection (Hocking and Pitt, 1984). Although different media have been used for HRM development, MEA (Malt Extract Agar) is the most commonly preferred. Other media used in different identification and isolation stages include CYA (Czapek Yeast Extract Agar), PDA (Potato Dextrose Agar), OMA (Oatmeal Agar), AJA (Apple Juice Agar), and OWA (Oatmeal Wheat Germ Agar) (Beuchat, 1986; Pitt and Hocking, 1984; Tournas, 1994; Rico-Munoz and Hobraken, 2014). To improve mould isolation, 50 mg/L rose bengal and 100 mg/L chloramphenicol are added to double-strength MEA (generally used for HRMs) Then equal amount of heat (75 C for 30min) applied food homogenate were mixed with the medium before plating. (Maneboon et al., 2023; Rico-Munoz et al., 2015). Other methods include filtration for liquid sugars, centrifugation, and the impedimetric method (impedimetry and conductimetry), which is used only for Byssochlamys (Kotzekidou, 2014).

There is a need for molecular-based biological methods that are fast and easy to identify moulds based on the genotype of the organisms. The first step in this process is generally accepted as DNA extraction. DNA isolation methods can vary due to the cell wall composition of moulds, which includes chitin, glucans, lipids, and other polymers resistant to enzymatic and chemical reagents. Various biochemical, mechanical, and physical methods, or combinations of these can be used for mould DNA isolation. Primary types of DNA extraction methods include lysis buffers containing SDS (dodecyl sulfate), proteinase K, SDS and lysis buffer, benzyl chloride, alkaline chemicals, cetyltrimethylammonium bromide, and DNA isolation kits. Mechanical methods combine shaking with glass beads (ceramic or silica) or mechanical homogenization. Physical methods include grinding with liquid nitrogen, microwaving, and using magnetic bead-based technologies. Especially for heat-resistant moulds like *Byssochlamys* and *Neosartorya* species, PCR (Polymerase Chain Reaction) methods are highly fast and useful for identification. Additionally, real-time PCR has been used for N*. fisheri* and *A. fumigatus* in various studies (Spiess et al., 2003; Weldhagen et al., 2008). RAPD (Random Amplified Polymorphic DNA) is another technique used to analyze genetic diversity among strains and mould populations. A previous study on *Neosartorya* species highlighted their similarity to *A. fumigatus* when RAPD-PCR was combined (Frac et al., 2015). Moreover, *Aspergillus* and *Penicillium* species can be identified using MALDI-TOF MS (Matrix Assisted laser desorption ionisation time of flight mass spectrometry) (Drissner and Freimoser, 2017; Normand et al., 2021).

Exo-Metabolites (Exstrolites) Produced by HRM

Enzymes

HRMs are considered critical microorganisms in the industry due to their production of various enzymes that degrade the structure of the product, leading to colony formation on the surface, phase separation, and cloudiness in beverages. Pectinases, amylases, cellulases, and proteinases produced by HRMs can cause food spoilage and economic loss (Salomão, 2018; Tournas, 1994). These enzymes also produce unpleasant odours, sour taste, and gas in foods (Tournas, 1994). Thus, more studies on the enzyme profiles synthesised by HRMs, which have limited literature, are needed.

Mycotoxins

It is known that heat-resistant moulds pose a health risk by producing various mycotoxins during their development in fruit products. For example, *Neosartorya spp*. produces fumitremorgins, fumitoxins, and verruculogen, while *Byssochlamys* produces patulin and byssochlamic acid, leading to food spoilage and mycelium formation (Houbraken et al., 2006; Puel et al., 2007). *Talaromyces* spp. synthesizes duclauxin and spiculisporic acid mycotoxins (Yamashita et al., 2019). Numerous studies addressing mycotoxins synthesized by HRMs can be found in the literature (Salomão, 2018; Kotzekidou, 2014; Lane, 2019; Piecková, 2020; Maj et al., 2023; Dijksterhuis, 2019; Kelfkens, 2024; Tournas, 1994). Mycotoxins produced by HRMs are shown in Table 4. Additionally, studies have emphasized the effects of patulin, produced by *Penicillium* and *Byssochlamys spp*., on human health. Patulin, primarily produced by *P. expansum* in apples, causes blue mould rot, leading to fruit loss. It has neurotoxic, immunotoxic, mutagenic, and carcinogenic effects, causing serious damage (Tournas, 1994; Samson et al., 2009; Salomão, 2018).

HRM Prevention Methods

To control HRM contamination in food processing facilities, high-quality raw materials must be used. To meet hygiene requirements, it is necessary to monitor, wash, and disinfect raw materials. Information on eliminating HRM ascospores from raw materials is limited. Since soil is the primary source of HRM ascospores, preventing soil contamination in raw materials through effective processes (such as rinsing and washing) is essential (Rico Munoz, 2017). In subsequent stages, the effective inactivation of HRMs can be achieved by using chlorine dioxide and acidified sodium chloride at adequate and correct concentrations. The concentration used generally varies depending on the microorganism load, species, and application method. Most studies focus on bacterial pathogens and non-heat-resistant Moulds. Dijksterhuis et al. (2018) evaluated the resistance of *Aspergillus* (*Neosartorya*), *Talaromyces*, and *Paecilomyces* (*Byssochlamys*) species to chlorine dioxide and iodine. The researchers reported that the most resistant species were *T. macrosporus* and *Paecilomyces variotii* (=*B. spectabilis*), surviving at 75 ppm chlorine dioxide concentration but not at 200 ppm. It was also found that the studied species survived at 75 ppm iodine concentration. It was determined that the inactivated spores did not germinate in the growth medium for 7 days. There is limited information regarding the elimination of HRM ascospores from packaging. Delgado et al. (2012) reported that the combination of 6-second heat treatment (70 °C) and hydrogen peroxide (35%) would provide sterilization when heat-resistant moulds such as *Aspergillus fischeri* and *P. variotii* are present with up to 1 spore per 100 cm^2. In addition to controlling temperature, time and chemical concentration, packaging must also be stored and handled hygienically. Eliminating HRM ascospores from beverage processing environments is generally challenging, as the highest ascospore concentrations are often found in areas cleaned using dry cleaning methods. These areas must be adequately cleaned and disinfected (Rico-Munoz, 2017). Acidified sodium chloride has been reported to be an effective disinfectant against mould spores (Johnson and Rico-Munoz, 2007). The Food and Drug Administration (FDA) has approved the use of 100-200 ppm sodium chloride for use on food-contact processing equipment (US-FDA, 2024). The Environmental Protection Agency (EPA) has approved the use of oxy-chlorine types for food-contact surfaces (US-EPA, 2024). The number of studies examining the effectiveness of disinfectants against HRM ascospores is quite limited, and more research is needed in this area. Strategies to prevent and combat HRM contamination can be summarized as follows: 1- Sanitation practices (such as GMP(Good Manufacturing Practices), GHP(Good Hygiene Practices), HACCP(Hazard Analysis of Critical Control Point)) and risk assessment, 2- Fruit selection (removal of damaged or spoiled products), 3- Use of chemicals (ClO2, iodine, chlorine-based disinfectants), 4- Combined methods (low water activity, pH, temperature), 5- Non-thermal inactivation methods (modified atmosphere packaging, high-pressure processing, pulsed electric fields) (Rico-Munoz and Dos Santos, 2019; Rico-Munoz et al., 2019; Groot et al., 2019; Stafenallo et al., 2020).

Table 4. Mycotoxins produced by HRM

Heat Resistant Moulds	Mycotoxins	References
Byssochlamys spp. (*Paecilomyces*)	Patulin, Byssotoxin A (Assimetrin and variotin) Byssochlamic acid, Byssochlamysol Mycophenolic acid, Viriditoxin	Houbraken et al. (2006) Puel et al. (2007) Salomão (2018) Kotzekidou (2014) Lane (2019)
Neosartorya spp. *(Aspergillus)*	Fumitremorgins (A, B and C), fumitoxins, verruculogen, Penitrem A, fischerin, fumigatin, fumagillin, tryptoquivalone, gliotoxin, fumigaclavines	Piecková et al. (2020) Lane (2019) Maj et al. (2023)
Talaromyces spp.	Duclauxin, spiculisporic acid, Avellanin A and B, emodin, vermiculin, vermicidin, vermistatin, talaromycin, trachysporic acid	Yamashita et al. (2019) Piecková et al. (2020)
Penicillium spp. *(Eupenicillium)*	Patulin, Ochratoxin A, Penicillic acid, Brefaldin A and C, Cupenifeldin (cyanein)	Salomão (2018) Piecková et al. (2020)

Conclusion and Recommendations

Due to the spoilage they cause in food, especially fruits, HRMs pose a hidden risk for the food industry. Preventing the formation of HRMs is becoming increasingly important due to their ability to produce mycotoxins and enzymes, and their ability to maintain activity at high temperatures. For this reason, thermal processes (such as pasteurisation, and thermisation) and non-thermal processes such as HPP, UV, TS, and MAP can be applied considering the variables of food brix°, pH, and aw. Additionally, it should be noted that intense heat treatment (>90°C), which inactivates ascospores and prevents spoilage, can also deteriorate the quality of the food (Dos Santos et al., 2018, Dos Santos and Membré et al., 2020). Moreover, selecting quality raw materials, HACCP, GMP, GHP, QMSRA applications are also important practices to prevent HRM contamination. Fruit products, which are frequently consumed and vary seasonally, are considered to be susceptible to soil-borne HRM contamination. Therefore, there is a need for comprehensive studies and efforts to prevent HRMs, rather than focusing on a single raw material or species.

Author contributions
All authors equally contributed

Conflicts of interest
The authors declare no competing interests.

Ethical Statement: The abstract of this article was published at the 4th International Euroasian Mycology Congress. It is hereby declared that scientific and ethical principles were followed during the preparation of this study and that all studies used were indicated in the bibliography**.**

It is declared that scientific and ethical principles have been followed while carrying out and writing this study and that all the sources used have been properly cited (Elif Doğan, Nükhet N. Demirel ZORBA).

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