Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Faculty of Natural Resources and Agricultural Sciences**

# Opportunistic pathogenic filamentous fungal species in health supplements – potential risk to immune compromised individuals

Opportunistiska filamentösa svamppatogener i hälsokosttillskott – risker för individer med nedsatt immunförsvar

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# Abstract

Invasive fungal infections (IFI) are a threat to individuals with a suppressed immune system and the rate of IFI cases in the world is increasing. These types of infections occur both at home and during hospitalization. At hospitals, small wounds caused by needles may later be infected with conidia, air-borne opportunistic pathogenic conidia may be inhaled, and contaminated food may be consumed. IFI in the gastrointestinal tract increases the mortality rate due to late detection of those infections. The objective of this study was to investigate if food products that immune suppressed individuals would prefer to buy, or products marketed toward those individuals, would contain any opportunistic pathogenic filamentous fungi. The study also compares if any difference can be found between samples incubated at the temperatures of 25 °C and 35 °C, this to simplify any screening of filamentous fungi. Among 16 different products (1 tea, 8 probiotics and 7 plant based products), filamentous fungi were only detected in two leaf powder products, one product of chia seeds, camomile tea, two probiotics and two fruit and vegetable products. The two opportunistic pathogenic species *Rhizopus oryzae* and *Mucor circinelloides* were detected in one of the probiotic products incubated at 35 °C. *R. oryzae* was also detected in chia seeds together with other opportunistic pathogens, such as *Lichtheimia ramosa* and *Aspergillus flavus* when incubated at 35 °C. Both *A. flavus* and *A. fumigatus* was detected in nettle leaf powder and fruit powder incubated at 35 °C. The fruit powder did however also contain the opportunistic pathogen *Byssochlamys spectabilis,* and the nettle leaf powder did also contain the opportunistic pathogen *R. microsporus*. Leaf powder made of *Moringa oleifera* was the only product that was found to be heavily contaminated with *L. ramosa.* The study could show that there is a slightly better chance to reduce the growth of non-opportunistic pathogenic species and target only the opportunistic pathogenic species by incubating samples at 35 °C. To completely avoid growth of non-opportunistic pathogenic species is not possible through this method only.



*Keywords:* IFI, opportunistic pathogen, immune compromised, food born disease, probiotic

## Sammanfattning

Mögelinfektion hos immunförsvagade patienter är en livshotande fara, och har visats ha en ökande trend i världen. Mögelinfektioner kan förekomma i hemmet och på sjukhuset via hål i huden från nålar som sedan kontamineras av mögelsporer, inandning av sporer i luften eller förtäring av kontaminerade livsmedel. Mögelinfektioner i mag- och tarmkanalen ger en risk för dödlig utgång på grund av att den ofta undkommer att identifieras. Syftet med denna studie var att undersöka om livsmedelsprodukter som personer med försvagat immunförsvar skulle tänka söka sig till, eller de produkter som marknadsför sig mot dessa individer, kan innehålla potentiella patogena mögelarter. Studien ska också jämföra om det förekommer några skillnader i mögeltillväxt av olika arter i prover som inkuberas i 25 °C eller 35 °C för att förenkla identifieringen av olika potentiella patogena mögelarter. Bland 16 olika livsmedelsprodukter (1 té. 8 probiotika och 7 produkter baserade på frukt och grönsaker) isolerades mögel från två bladpulversprodukter, chiafrön, kamomill té, två probiotikaprodukter och två frukt- och grönsakspulvriserade produkter. De potentiella patogena mögelsvamparna *Rhizopus oryzae* och *Mucor circinelloides* var påvisad i en av probiotikaproverna som inkuberades i 35 °C. Förutom i probiotika fanns även *R. oryae,* bland de potentiella patogena arterna *Lichtheimia ramosa* och *Aspergillus flavus* i chiafrön inkuberade i 35 °C. Både *A. flavus* och *A. fumigatus* kunde påvisas i både bladpulver från nässla och i fruktpulver inkuberade i 35 °C. Fruktpulvret visades även innehålla spår av den potentiella patogena mögelarten *Byssochlamys spectabilis,* och nässelbladpulvret påvisades ha spår av *R. microsporus.* Bladpulver gjord av *Moringa oleifera* var den enda produkten som visades innehålla flest kolonier av mögelarten *L. ramosa.* Studien kunde visa att vissa oönskade icke-potentiella patogena mögelarter minskade på de plattor som inkuberades i 35 °C. Men att nå en fullständig isolering av endast potentiella patogena mögelarter och undgå andra icke-potentiella patogena mögelarter går inte att genomföra med endast denna metod.



*Nyckelord:* mögelinfektion, potentiella patogena, immunförsvagad, livsmedelsburen sjukdom, probiotika

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# Abbreviations

| | |
|---|---|
| IFI | Invasive fungal infection |

# 1 Introduction

The immune system can be ineffective among elderly people or infants, and also due to malnutrition, genetic defects, infections that suppress the immune system (similarly to AIDS) and in patients going through treatments that suppress the immune system (Nandini, 2005; Nissen et al. 1999). Immune suppressed people, whatever the cause, will be more vulnerable to exposure of pathogenic bacteria or fungi. Furthermore, this exposure can in many cases be difficult to avoid because of the environment usually being rich in these two types of microorganisms, indoors as well as outdoors. In our environment there will always be fungus spores in contact with people in various ways. Spores of many species are inhaled, or consumed through contaminated products. Some fungal species are beneficial for us in production of beverages, conserving food, enhancing the taste of food and being used for medicinal preparation. However, there are also harmful species, producing toxins, giving allergic reactions or potentially causing mycosis (Benedict et al., 2016).

Systemic mycosis, or invasive fungal infection (IFI), has by several studies been found among immune suppressed people and is according to Brenier-Pinchart et al. (2006) feared to afflict patients in the haematological unit, treated with chemotherapy or going through bone marrow transplantation. Even if bacterial infections are more common in comparison with fungal infections, there has been a trend with increasing fungal infection by *Aspergillus* and gastrointestinal mucormycosis (Erjavec et al., 2009; Spellberg, 2012). The increasing amount of IFI is believed to correlate with the increasing cases of cancer, a population that is older and other diseases as obesity, diabetes and gastrointestinal inflammations requiring corticosteroid therapy. A worrying resistance to azoles, commonly used anti-fungal agents, among *Aspergillus fumigatus* has been found, arisen because of the heavy use of fungicides in the agricultural production (Vermeulen et al., 2013). This can lead to a global problem in the future.

The fact that the fungal spores are mostly airborne results in different challenges to avoid fungal contamination compared to bacteria. The most common way for fungal infections to develop is through inhalation of conidia or entering the body through surgical stitches or other types of breaches in the skin (Bouakline et al., 2000). Several studies have indicated that one route of mycosis infection is derived

from food contaminated with spores, which in turn leads to IFI in the gastrointestinal tract (Benedict et al., 2016; Martino and Subirà, 2002). The severity of those cases can most of the time be fatal due to a high risk of not detecting the infection in time (Dioverti et al. 2015). It is hard to determine the source of the conidia that contaminates the food, but it is suspected that it is a combination of conidia already present in the hospital and conidia contaminating the food during the processing of the food products. In one case, studying the aspergillosis cases in a hospital in relation to detected *Aspergillus* in air it was shown that if the hospital environment undergoes a reduction of airborne *Aspergillus* conidia contamination, one can successfully reduce the fungal infections among immune suppressed patients (Arnow et al., 1991).

The yeast *Candida* and the filamentous fungi *Aspergillus*, and *Rhizopus* and other *Mucormycetes* are pathogens that can cause infections resulting in death for immunocompromised patients (Benedict et al., 2016; Brenier-Pinchart et al., 2006; Tomsikova, 2002). These filamentous fungi can frequently be found on various food products, and can be a problem for those patients that need to avoid food products contaminated with any conidia. There is a possibility for the food industry to offer the immunocompromised consumer group food that is free from potentially pathogenic fungi. This would be achieved by scanning their products for any fungal contamination, but the traditional fungal analysis to identify potentially pathogenic species is time consuming and financially ineffective leading to many companies not performing this analysis, although many companies will perform general yeast and mould counts as part of their hygiene analysis. This study will focus on reviewing cases of IFI that are caused by consuming food products, analysing dietary supplements and food products that immunocompromised persons would possibly choose, or that are intended to boost the immune system of the consumer. The samples are incubated at both 25 °C and 35 °C and it is reviewed if there is a possibility to within 7 days perform a food scan that can select for opportunistic pathogenic fungi and have preliminary identification performed by an untrained person.

# 2 Background

## 2.1 Literature review of IFI cases

A few studies world-wide can be found identifying food as the cause of IFI in the gastrointestinal tract. However, the majority of the studies do not tell what type of food source the fungal contamination could derive from (table 1).

Table 1. *IFI case studies with fungal infection in the gastrointestinal tract, source of infection, their outcome and the name of the fungi.*

| Study | Fungi name | Outcome | Reference |
|---|---|---|---|
| 8-year retrospective study of autopsy | *Aspergillus* | 34/107 cases located in the gastrointestinal tract | (Kami et al., 2002) |
| **Source (confirmed or postulated source)** | **Fungi name** | **Outcome** | **Reference** |
| Fermented corn beverage | Mucormycete | Died | (Benedict et al., 2016) |
| Naturopathic medicine | *Mucor indicus* | Survived | |
| Cereal | *Fusarium moniliforme* | Survived | |
| Unrefrigerated meal | *Mucor indicus* | Survived | |
| Dried salted fish | *Monascus ruber* | Died | |
| Home-brewed beer | *Rhizopus* sp. | Survived | |
| Yogurt | *Mucor circinelloides* | Died | |
| Dietary supplement | *Rhizopus oryzae* | Died | |
| Cheese | *Candida catenulata* | Died | |
| Vacuum flask of milk | *Blastoschizomyces capitatus* | One died and one survived | |

Table 1 (continuing). *IFI case studies with fungal infection in the gastrointestinal tract, source of infection, their outcome and the name of the fungi.*

| Source (confirmed or postulated source) | Fungi name | Outcome | Reference |
|---|---|---|---|
| Unknown | *Rhizopus microsporus* | Died | (Cheng et al., 2009) |
| Unknown | *Rhizopus microsporus* | Survived | |
| Unknown | *Rhizopus microsporus* | Died | |
| Unknown | *Rhizopus microsporus* | Died | |
| Unknown | *Rhizopus microsporus* | Died | |
| Unknown | *Rhizopus microsporus* | Survived | |
| Unknown | *Rhizopus microsporus* | Survived | |
| Unknown | *Rhizopus microsporus* | Died | |
| Unknown | *Rhizopus microsporus* | Survived | |
| Unknown | *Rhizopus microsporus* | Survived | |
| Unknown | *Rhizopus microsporus* | Survived | |
| Unknown | *Rhizopus microsporus* | Survived | |
| Unknown | *Mucormycosis* | 16/28 died | (Dioverti et al., 2015) |
| Unknown | *Rhizopus* spp. | Died | (Kalva et al., 2013) |
| Unknown | *Mucormycosis* | Survived | (Goel et al., 2013) |

In an 8-year retrospective study of autopsy, reviewed by Kami et al. (2002), 11.4 % (119 of 1043) of all autopsy subjects were infected with invasive aspergillosis. Of the infected patients, 31 % had the infection located in the gastrointestinal tract, but if it was caused by ingestion of contaminated food or infection through usage of contaminated tongue depressor is not determined. In the study it was also mentioned that the infection could have been spread from the lung to the gastrointestinal tract.

In general, IFI are mentioned by several authors to be a major cause of mortality among patients with weak immune systems, especially among patients going through organ transplantation and stem cell transplantation. According to Pappas et al. (2010), the survival rate within one year among patients with IFI, going through an organ transplant, is 59 %. The survival rate for patients receiving stem cell transplants is 25.4 % (Kontoyiannis et al., 2010). For all the stem cell transplant patients, 43 % of the IFI was caused by aspergillosis, and among organ transplant patients 19 % was caused by aspergillosis (see figure 1). Among the organ transplantation patients, the patients with a small bowel transplant had the highest incidence rate of IFI. The same study also shows that 25 % of all IFI cases have developed 3 years after the transplantation, indicating a late-occurring IFI is possible for those patients.

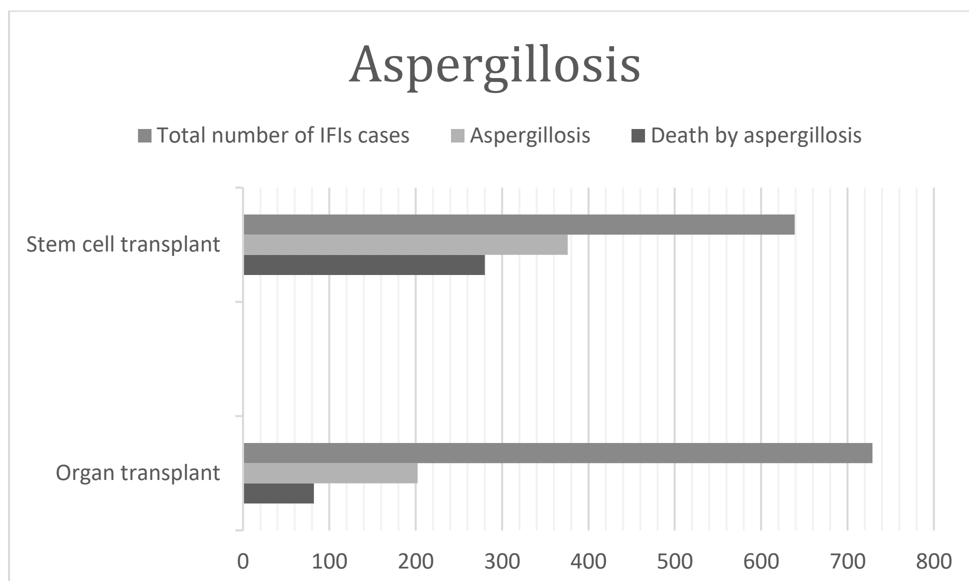


Figure 1. *Number of cases and outcome for patients going through stem cell transplantation (Pappas et al., 2010) and organ transplantation (Kontoyiannis et al., 2010) that are affected by invasive* Aspergillus *infection.*

The total number of cases accounts for 3.9 % of the total number of patients going through stem cell transplantation and 4.3 % of organ transplant patients during the years 2001 to 2006.

## 2.2 Literature review of opportunistic pathogenic filamentous fungi

### *2.2.1 Aspergillus*

*Aspergillus* belongs to the family *Ascomycota* and includes more than 180 species, a few of them being very common contaminants in both our food and environment (Samson et al., 2004). *Aspergillus* is more commonly found in subtropical and tropical climates compared to the climate in northern Europe. It grows well even in conditions of low water activity and high acidity. *A. fumigatus* is found to be the most pathogenic species of *Aspergillus* and can grow at a temperature above 50 ℃.

Vallabhaneni et al. (2015) presents that 20 % of all IFI in transplant patients in USA was due to *Aspergillus* infections. One French study included in the review by Vallabhaneni et al. (2015) could show an increasing trend of invasive aspergillosis by 4.4 % each year when studying the years between 2001 and 2010. The authors discussed that similar trends can be found in other parts of the world, but there is not sufficient data to determine the global burden of aspergillosis. The same study does not mention whether or not the IFI are caused by contaminated food, neither if they are located in the gastrointestinal tract. As mentioned above, in an 8-year retrospective study performed by Kami et al. (2002) it was found that 11.4 % of all deaths of patients in the haematological malignancy unit were caused

by invasive aspergillosis, and 31.7 % of these *Aspergillus* infections were located in the gastrointestinal tract. *Aspergillus fumigatus* is the most common species in the *Aspergillus* family to cause infections in humans, and is suggested by Latgé (2001) to be responsible for over 90 % of all cases of aspergillosis located in the lung.

### 2.2.2 Fusarium

Just like *Aspergillus, Fusarium* belongs to the family *Ascomycota*. *Fusarium* is commonly seen as a plant pathogen, especially in cereals, and is found in all regions of the world. Few species of *Fusarium* can be pathogenic and grow at temperature above 37 °C. (Samson et al., 2004)

Invasive *Fusarium* infections has been shown to have an incidence rate of 0.25 % in the US, and a mortality rate ranging from 50 % to 80 % (Vallabhaneni et al., 2015). The same study does not mention where the infection is located or where it is derived from. In a case from USA from 1995, the tap water was identified as a source for *Fusarium* contamination in a hospital. This resulted in 3.8 cases of IFI per year between 1975 and 1995 (Dignani and Anaisse, 2004). The survival rate of *Fusarium* IFI according to a cohort study conducted by Kontoyiannis et al. (2010) is 6.3 %.

### 2.2.3 Mucormycetes

*Mucormycetes* includes several species in the family Zygomycetes and are divided into *Lichtheimia (Absidia), Mucor, Rhizopus* and *Syncephalastrum. Lichtheimia* and *Rhizopus* are common contaminants in both air and on food such as fruit, grains and vegetables. Both *Rhizopus* and *Mucor* are used in fermented food production. *R. oryzae* is shown to be the most common species of the family Zygomycetes to cause mucormycosis according to Petrikkos et al. (2014).

Mucormycosis is the second most common IFI that is caused by filamentous fungi such as *Rhizopus*, *Lichtheimia*, *Mucor* or *Rhizomucor*. Yet, it is much less common than aspergillosis (Vallabhaneni et al., 2015; Dioverti et al., 2015; Nissen et al., 1999). An increasing trend has been found of mucormycosis, suspected to correlate with the increasing rates of obesity, diabetes and problems with inflammation in the gastrointestinal tract (Spellberg, 2012). Data from five studies of mucormycosis cases between the years 1885 to 2009 could show that *Rhizopus* is the most common infectious agent causing mucormycosis and reported cases have increased after the year 1980 (Petrikkos et al., 2012). In a study carried out by Cheng et al. (2009), the authors investigated gastrointestinal infections and mucormycosis of *Rhizopus microsporus*. Furthermore, it has been mentioned that there is a high risk of mortality in these types of infections, especially among premature neonates and neutropenic adults when reviewing studies that investigated these groups of people (Nissen et al., 1999; Siu and Lee, 2004). A high mortality rate among patients suffering from gastrointestinal mucormycosis can also be seen in the study by Pagano et al. (1997). The high mortality rate caused by mucormycosis, or other invasive fungal infections, is in several studies

determined to be caused by late diagnosis because of the difficulties to detect it, and by the usually low suspicion that one is infected by an invasive fungus (Petrikkos et al., 2012; Dioverti et al., 2015). This, according to the authors, increases the mortality rate up to 85 %.

## 2.3 Literature review of common fungal contamination in hospital food

Two studies analysing different food products that are usually handled in hospitals have been found in the literature. Only one of these studies provides the reader with the full names of the fungal species (table 2).

Table 2. *Food product analysed by other studies, number of samples, percentage of contamination and the name of fungi found.*

| Food product | Number of samples | % contamination | Fungus | Reference |
|---|---|---|---|---|
| Pepper | 15 | 100 | *A. fumigatus, A. flavus* and *Mucorales* | (Bouakline et al., 2000) |
| Regular tea | 15 | 100 | *A. fumigatus* and *A. niger* | |
| | 15 | 33 | *Mucorales* | |
| Herbal tea | 22 | 27 | *Mucorales* | |
| Exotic fruit | 42 | 50 | *A. fumigatus* | |
| | 42 | 51 | *A. niger* | |
| | 42 | 29 | *Trichoderma* spp. | |
| Apple and orange | 30 | 20 | *A. fumigatus* | |
| Fruit | 13 | 38 | *Penicillium* sp., *Acremonium* sp. and *Cladosporium* spp. | (Brenier-Pinchart et al., 2006) |
| Honey | 7 | 3 | *A. niger, Penicillium* spp. and *Zygomycetes* | |
| Cream cheese | 20 | 15 | *A. terreus, Penicillium* spp. | |
| Pasta and rice | 22 | 9 | *Aspergillus* sp. and *Penicillium* spp. | |
| Cooked vegetables | 77 | 9 | *A. fumigatus, Aspergillus* spp. and *Penicillium* spp. | |
| Fruit juice | 14 | 7 | *Penicillium* spp. | |
| Cooked meat and fish | 98 | 6 | *Penicillium* spp., *Alternaria* spp. and *Cladosporium* spp. | |

According to both Bouakline et al. (2000) and Brenier-Pinchart et al. (2006) it is clear that *Aspergillus* is the most common contaminant in all different food products analysed. When *A. fumigatus* was present it had usually contaminated the

majority of the samples and could be found in pepper, tea, vegetables, exotic fruits and regular fruits such as apples and oranges.

## 2.4 Food to avoid by patients in risk groups

The food prepared for the histological unit for immunosuppressed patients at the Uppsala University Hospital in Sweden, are delivered by the company Kost, located in Västerås (Interview, 2016a). There are a few restrictions in the hospital considering the handling of food products and which products are allowed in the hospital (Interview, 2016b). The most important restrictions are: all food products must be heated to reach a temperature of 72°C, all taps must run for 10 to 15 seconds before filling a glass with water, and all vegetables must be peeled and cleaned with clean tap water. Food products not allowed are listed in table 3.

Table 3. *Food products not allowed in the histological unit of the Uppsala University Hospital.*

| **Product category** | **Food products** |
| --- | --- |
| Sea water products | Pickle raw salmon or other meat, fresh shellfish, smoked fish or sushi |
| Meat | Air dried meat |
| Vegetable and fruit | Raw raisin, plum and unpeeled vegetable |
| Pre-packaged products | Ready to eat food which are not intended to be heated such as sandwich, bagel, mayonnaise, salad, pizza and hamburger |
| Milk based products | Mould cheese, any type of ice-cream and milk products with living microorganisms |
| Other | Candy, ready to eat products that are vacuum packaged and fresh spices, for example dill and basil |

All food products mentioned in table 3 are susceptible to contamination by microorganisms such as bacteria or fungal conidia. All these products have in some way been in direct contact with the environment, not sealed for a longer time and not been treated to eliminate any hazards as bacteria or conidia. According to Tomblyn et al. (2009), food in general should go through any type of heat treatment such as cooking or roasting to reach an inner core temperature above 72°C. The food should be packaged in a sterile container, such as canned food products, or be directly served. Concerning raw fruits, it is safe to peel them and wash them well before serving.

# 3 Methods and Materials

## 3.1 Literature search

All scientific literature used in this study are those that were accessible through the search engine “Primo” on the SLU libraries website. The use of the search engine was limited because SLU does not subscribe to certain scientific journals. The literature search was done in English, since almost all studies are written in English. The keywords used were “mucormycosis”, “invasive fungal infection”, “gastrointestinal infection” together with “food”, “hospital”, “immunosuppressed” or “immune compromised”.

## 3.2 Interview

An interview with a nurse at the haematology unit was performed via e-mail. Another interview was performed with the food safety team leader at the company Kost in Västerås. Kost delivers all food to the haematology unit at the hospital.

## 3.3 Fungal isolation

All fungal isolation and identification was performed at the Swedish University of Agricultural Sciences (SLU) in Uppsala. Plating and all work with products was performed at a sterile bench with ventilation (laminar flow hood) to avoid any accidental contamination. All products were resealed with a sterile plastic bag after the product package was opened and samples taken from it. Sterile peptone water (0.1% w/v) was used if necessary for those products that needed to be blended in the stomacher before plating. The isolation medium used was malt extract agar with chloramphenicol (0.01% w/v) (MEAC) to inhibit bacterial contamination. The MEAC agar and peptone solution were prepared with distilled water and autoclaved before use. All equipment and benches were sterilized with 70 % ethanol solution before coming in contact with the samples.

For incubation, a room with a temperature of 25℃ and a smaller incubation chamber with a temperature of 35℃ were used. The 35℃ incubation chamber is

completely dark, whereas there is some light in the room with a temperature of 25ºC.

All fungi growing on the samples in 35ºC were purified by inoculating at three points on the MEAC plate and incubated in 25ºC for at least 7 days before identification.

## 3.4 Fungal identification

The fungal identification of all samples was performed with the help of a microscope and the textbook Introduction to food- and airbourne fungi (Samson et al., 2004). All samples incubated at 35 ºC were purified and grown in a separate plate at 25 °C for 7 days, after which DNA was extracted by the method of Cenis (1992). The primers used in all samples for the PCR were ITS1F/ITS4 amplifying the international transcribed spacer (ITS), except the samples with suspected *Penicillium*. For *Penicillium* samples, primers bt2a / bt2b amplifying a section of the beta-tubulin gene were used. For both primers sets, the following cycling parameters were used: 94 °C for 1 minute, 50 °C for 1 minute, 68 for 2 minutes and run for 35 cycles. The final step was 72 °C for 5 minutes. The purification of PCR products and sequencing was performed by Macrogen Europe and after that analysed in BLAST. With BLAST any unknown sequence can be compared against a database of sequences to search for the identity of the fungi. Any results with poor sequences were re-amplified and re-sequenced.

## 3.5 Product tested

All products tested in this study were bought at the stores Life and Hemköp in Uppsala and were stored at an ambient temperature. No products were sensitive to the temperature interval between 10ºC and 30ºC. Only single aliquots of each product were analysed, however isolations were performed on several replicate plates. All products used are presented in table 4.

Table 4. *General information about the products tested in this study, their ingredients, where they are produced and any additional information that can be of any importance in this study.*

| Product name | Ingredients | Production | Additional information |
|---|---|---|---|
| Camomile tea | Dried camomile tea in tea bags | Agriculture outside of EU | EU-organic |
| Aloe Vera juice | 99.8 % cold pressed Aloe Vera (*Aloe barbadensis* Miller*)* and 0.2 % lemon juice | Agriculture in Mexico and gently pasteurized | EU-organic |
| Chia seeds | Chia seed "*Salvia hispanica*" | Not agriculture in EU | EU-organic |
| Raw Nettle leaf powder | Nettle leaf powder | Northern Europe | EU-organic |
| Morning leaf powder | Morning leaf "*moringa oleifera*" | Agricultured in India | EU-organic |
| Super booster fruit powder (no production or additional information) | 51 % fruit (apple fiber, rose hip, acerola, grape seed extract, pomegranate extract, cranberry, acai berries, blueberries, goji berries, strawberries, raspberries, papaya and pineapple), 28.5 % greens (barley, grass, wheat, spirulina and chlorella), remainder is spinach, carrot, red beet, tomato, brussels sprout, broccoli sprout and sweeteners. | | |
| Super greens | 60 % wheatgrass, 35 % spirulina, 5 % mint | Not declared | EU-organic |
| Bio-Strath | 89 % yeast fermented herbs (*Saccharomyces cerevisiae*) | Made in Zurich, Switzerland | In tablet form |
| Pure Biotic | *Lactobacillus* and *Bifidobacterium* | Made in Canada | Marketed to children from 3 years old, tablet form |
| Probi järn (probi iron) | *Lactobacillus* | Made in Lund, Sweden | Powder form in a capsule |
| Probi frisk (probi well) | *Lactobacillus* | Made in Lund, Sweden | In tablet form |
| Probi mage (probi stomach) | *Lactobacillus* | Made in Lund, Sweden | Powder form in a capsule |
| Propex express | *Siberian ginseng,* cellulose and *Malpighia punicifolia L.* | Made in Belgium | In tablet form |
| Probiotika life | *Lactobacillus* and *Bifidobacterium* | Made in Finland | Powder form in a capsule |
| Lacti Plus | *Lactobacillus* and *Bifidobacterium* | Made in Canada | Powder form in a capsule |
| Probioplex | *Streptococcus, Lactobacillus* and *Bifidobacterium* | Not declared | Powder form in a capsule |

Most of the products presented in table 4 are certified as EU-organic (meaning they follow the European legislation for organic agricultural production (SLV, 2016)) and cultured outside of northern Europe. There is a mix of products from

probiotics to whole seeds and powdered form of fruit and vegetables analysed for fungal contamination. Super booster fruit powder is the only product containing more than five ingredients and contains fruits, cereals and vegetables.

## 3.6 Homogenisation and plating procedure for all products

The procedure for the products varied depending on the texture of the product. The pre-process and plating used for each product are presented in table 5. In direct plating, the product was either pipetted directly to the plate if it was in a liquid state or just sprinkled on to the medium if it was in a solid and powdery from. All products such as tablets were crushed with a sterile mortar and pestle before being sprinkled on to the medium.

Table 5. *Product tested, processing method, plating method and incubation temperature.*

| Product name | Pre-process | Type of plating | 25 °C | 35 °C |
|---|---|---|---|---|
| Camomile tea | - | Direct plating 0.2 g | 5 | 5 |
| | 10 g tea held for 5 minutes at 90 ℃ of 90 ml peptone water | Direct plating of 3 g to 4 g mixture | 2 | 3 |
| Aloe Vera juice | - | Direct plating of 0.1 ml | 5 | 5 |
| | 1 ml Aloe Vera mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 5 | 5 |
| Chia seeds | 10 g seeds mixed with 90 ml peptone water in a stomacher | Direct plating of 0.6 g to 2 g mixture | 7 | 7 |
| Moringa leaf powder | - | Direct plating 0.3 g to 0.4 g | 5 | 5 |
| | 1 g mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 2 | 2 |
| Super booster fruit powder | - | Direct plating 0.3 g to 0.5 g | 5 | 5 |
| | 1 g mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 2 | 2 |
| Raw nettle leaf powder | - | Direct plating 0.3 g to 0.5 g | 5 | 5 |
| | 1 g mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 2 | 2 |
| Super greens | - | Direct plating 0.2 g to 0.5 g | 5 | 5 |
| | 1 g mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 2 | 2 |
| Bio-Strath | 1 tablet mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 5 | 5 |
| Pure Biotic | 1 tablet mixed with 9 ml peptone water | Dilution plating of 0,1 ml of 1:10 solution | 5 | 5 |

Table 5 (continuing). *Product tested, processing method, plating method and incubation temperature.*

| Product name | Pre-process | Type of plating | 25 °C | 35 °C |
|---|---|---|---|---|
| Probi järn (probi iron) | - | Direct plating of 1 capsule | 5 | 5 |
| Probi frisk (probi well) | 1 tablet mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 5 | 5 |
| Probi mage (probi stomach) | - | Direct plating of 1 capsule | 5 | 5 |
| Propex express | 1 tablet mixed with 9 ml peptone water | Dilution plating of 0.1 ml of 1:10 solution | 5 | 5 |
| Probiotika life | - | Direct plating of 1 capsule | 5 | 5 |
| Lacti Plus | - | Direct plating of 1 capsule | 5 | 5 |
| Probioplex | - | Direct plating of 1 capsule | 5 | 5 |

Tablets and other dry products except powdered probiotics were pre-processed by diluting them in peptone water before plating. Chia seed had to be soaked in peptone water for 30 min before homogenisation in a stomacher. The tablets were crushed with a sterile mortar and pestle and were dissolved in peptone water for 1 hour before plating. All probiotics that were in a powdered form in capsules was opened above the plate to collect all powder on the plate, after which the powder was spread with a sterile spreader.

# 4 Results

## 4.1 Incubation results

The results for each product from the incubation at different temperatures are presented in table 6.

Table 6. *Product tested, type of plating and number of positive and negative result (number of plates showing fungal growth).*

| Product name | Type of plating | positive result | negative result | Incubation temperature |
|---|---|---|---|---|
| Camomile tea | Direct plating | 1 | 6 | 25 ºC |
| | | | 7 | 35 ºC |
| | Direct plating from heated tea bag | | 7 | 25 ºC |
| | | | 7 | 35 ºC |
| Aloe Vera juice | 0.1 ml direct plating | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| | 0.1 ml of 1:10 dilution plating | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| Chia seeds | Direct plating of mixture from 1:10 solution | 7 | | 25 ºC |
| | | 7 | | 35 ºC |
| Raw nettle leaf powder | Direct plating | 5 | | 25 ºC |
| | | 5 | | 35 ºC |
| | Dilution plating from 1:10 solution | 2 | | 25 ºC |
| | | 2 | | 35 ºC |
| Super booster fruit powder | Direct plating | 3 | 2 | 25 ºC |
| | | 2 | 3 | 35 ºC |
| | Dilution plating from 1:10 solution | 1 | 1 | 25 ºC |
| | | | 2 | 35 ºC |

Table 6 (continuing). *Product tested, type of plating and number of positive and negative result (number of plates showing fungal growth).*

| Product name | Type of plating | positive result | negative result | Incubation temperature |
|---|---|---|---|---|
| Morning leaf powder | Direct plating | 2 | 3 | 25 ºC |
| | | 2 | 3 | 35 ºC |
| | Dilution plating from 1:10 solution | 1 | 1 | 25 ºC |
| | | 2 | | 35 ºC |
| Super greens | Direct plating | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| | Dilution plating from 1:10 solution | | 2 | 25 ºC |
| | | | 2 | 35 ºC |
| Bio-Strath | Dilution plating from 1:10 solution | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| Pure Biotic | Dilution plating from 1:10 solution | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| Probi järn (probi iron) | Direct plating | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| Probi frisk (probi well) | Dilution plating from 1:10 solution | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| Probi mage (probi stomach) | Direct plating | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| Propex express | Dilution plating from 1:10 solution | | 5 | 25 ºC |
| | | | 5 | 35 ºC |
| Probiotika life | Direct plating | | 5 | 25 ºC |
| | | 1 | 4 | 35 ºC |
| Lacti Plus | Direct plating | 3 | 2 | 25 ºC |
| | | 2 | 3 | 35 ºC |
| Probioplex | Direct plating | | 5 | 25 ºC |
| | | | 5 | 35 ºC |

The majority of samples had no growth of fungi on them. The analyses were only conducted on single samples of each product but with several replicate plates. Most probiotic samples did not display any positive results except for Lacti plus and Probiotika life. Chia seed, Raw nettle leaf powder and Morning leaf powder were the most heavily contaminated products. Second most heavily contaminated was Super boost fruit powder and the least contaminated product of those giving positive results was the camomile tea.

## 4.2 Identification results

The fungi identified and their description from each positive result shown in table 6 are listed in tables 7 to 12. All results from the 25 ºC incubation were identified with help from the textbook by Samson et al. (2004). All results from the 35 ºC

incubation were purified and incubated again at 25 °C and thereafter sent for identification by PCR and sequencing. The focus on accurate species identification only for fungi from the 35 °C incubation was because those can be potentially pathogenic species capable of growing at our body temperature.

### 4.2.1 Camomile tea

Table 7. *Result for tea, type of plating, weight of sample, name of fungi identified in microscope, incubation temperature in* °C *(inc. temp.), number of plates (contaminated/total) and size of the fungi in cm presented.*

| Product name | Type of plating | Weight | Inc. Temp. | Colour and size of fungi on primary isolation plates | Number of plates | Fungi identified |
|---|---|---|---|---|---|---|
| Camomile tea | Direct | 0.1 g | 25 °C[a] | 5-6 cm. White and randomly pink parts in the middle. | 1/7 | *Ascomycetes* |

[a] 0-3 colonies per plate.

One colony did grow on one of the 7 plates of camomile tea. The fungi were identified with microscope as an Ascomycete. The fungi were incubated for 7 days, which resulted in a very fluffy and flat appearance. In microscope, it was clearly visible that it had high numbers of septa and branches of small mycelium and square formed conidia. Brown ascoma were also visible, making it more probable that the fungus belonged in the family of Ascomycetes, based on the examined colony having identical microscopically shapes and details to Ascomycetes (Samson et al., 2004). The colony could not grow when inoculated on a new plate and incubated at 35 °C.

### 4.2.2 Raw nettle leaf powder

Table 8. *Result for raw nettle leaf powder, type of plating, weight of sample, name of fungi identified in microscope (PCR identification for 35 ºC), incubation temperature in* ºC *(inc. temp.), number of plates (contaminated/total) and size of the fungi in cm presented.*

| Product name | Type of plating | Weight | Inc. Temp. | Colour and size of fungi on primary isolation plates | Number of plates | Fungi identified |
|---|---|---|---|---|---|---|
| Raw nettle leaf powder | Direct | 0.3 g to 0.5 g | 25 ºC[d] | 4-5 cm. Olive green, white margins and yellow/white spots. White in the middle. | 5/5 | *Aspergillus oryzae* |
| | | | | >8 cm. Grey/white with black spots. | 5/5 | *Rhizopus stolonifer* |
| | | | 35 ºC[a] | >8 cm. Black/grey. | 5/5 | *Rhizopus oryzae* |
| | 1:10 dilution | 1 g | 25 ºC[b] | 4-5 cm. Black with white margins. | 2/2 | *Aspergillus niger* |
| | | | | 2-4 cm. Olive green/yellow in middle, large white margins. | 2/2 | *Aspergillus flavus* |
| | | | | 1-3 cm. Green/dark blue and little white in middle and white magrins. | 2/2 | *Aspergillus fumigatus* |
| | | | 35 ºC[a] | >8 cm. White/grey. | 2/2 | *Rhizopus microsporus* |
| | | | | 1-3 cm. Green/dark blue and little white in middle and white margins. | 2/2 | *Aspergillus fumigatus* |
| | | | | 2-4 cm. Olive green/yellow in middle, large white margins. | 2/2 | *Aspergillus flavus* |

[a] 0-3 colonies per plate, [b] 3-6 colonies per plate, [d] more than 10 colonies per plate.

All plates with raw nettle leaf powder showed results indicating that the product was heavily contaminated. All direct plated plates incubated in 25 °C had more than 10 colonies of different species growing on them, resulting in most of the fungi merging together and becoming harder to isolate and identify. One fungus had a clearly fluffy appearance with small yellow structures reminding of seeds close to the middle and was identified as *Aspergillus oryzae*.

All dilution plated plates incubated in 25 °C had between five and six colonies, some of them merged together but still distinguishable and possible to isolate and identify. *Aspergillus* spp. were dominating the plate and those identified were *A. niger, A. flavus* and *A. fumigatus.* These species were easily identified by their colour and appearance on the plates. *A. niger,* having a flat shape and completely black colour, was the only *Aspergillus* species that was not present on any plates incubated in 35 °C. PCR identification on those colonies growing in 35 °C could

verify *A. fumigatus* and *A. flavus*. There were fewer colonies growing at 35 °C than in 25 °C and *Rhizopus microsporus* did appear on all the plates, covering the whole plate and having a flat appearance. Both *A. flavus* and *A. fumigatus* were flat, yet *A. flavus* has a slightly lifted appearance in the middle of the colony.

Another fungus was identified by microscopy as *Rhizopus stolonifer* due to its almond-shaped sporangiospores, brown stipes and black zygospores. *R. stolonifer* and *A. oryzae* were also undoubtedly present on all plates at 25 °C, but appeared to be absent on plates incubated in 35 °C. The colonies incubated in 35 °C on direct plated plates were reduced so that they consisted of one to three colonies per plate, of which most of them had merged together and were identified by PCR as *Rhizopus oryzae*.

### 4.2.3 Morning leaf powder

Table 9. *Result for morning leaf powder, type of plating, weight of sample, name of fungi identified in microscope (PCR identification for 35 °C), incubation temperature in °C (inc. temp.), number of plates (contaminated/total) and size of the fungi in cm presented.*

| Product name | Type of plating | Weight | Inc. Temp. | Colour and size of fungi on primary isolation plates | Number of plates | Fungi identified |
|---|---|---|---|---|---|---|
| Morning leaf powder | Direct | 0.3 g to 0.5 g | 25 °C[a] | >8 cm. Dark grey on margins and light black/white in middle. | 2/5 | *Lichtheimia* sp. |
| | | | 35 °C[a] | >8 cm. Dark grey on margins and light or dark/grey in middle. | 2/5 | *Lichtheimia ramosa* |
| | | | | 25 °C: >8 cm. White/light grey. | | |
| | 1:10 dilution | 1 g | 25 °C[a] | 5 cm. Grey. | 1/2 | *Lichtheimia* sp. |
| | | | 35 °C[a] | 1 cm. White. | 1/2 | *Penicillium camemberti* |
| | | | | >8 cm. Light grey. | 1/2 | *Lichtheimia ramosa* |

[a] 0-3 colonies per plate.

On direct plated plates incubated at both 25 °C and 35 °C, similar species of Zygomycetes were visible. Two of five plates had between two to three colonies growing and all were completely covered with mycelium to the top of the lid. There was no difference in colour when incubated in 25 °C or 35 °C and in microscope both had small columellae with bent sporangiophores giving a branching look to the mycelium. By microscope the Zygomycetes were identified as *Lichtheimia ramosa* and supported by the PCR identification of those colonies growing in 35 °C.

Dilution plated plates contained species of Zygomycetes similar to those growing on direct plated plates. The result of the microscopy was also similar to the direct plated plates, with similar microscopic structures, which suggested that the

colonies consisted of *Lichtheimia corymbifera*. Although, through PCR identification it was confirmed that it was more likely to be *Lichtheimia ramosa*.

On one of the plates incubated in 35 ºC, a small fungus with a fluffy appearance and raised, hill like structure was growing. Its microscopic structure consisted of branches with metulae, phialides and a rough stipe. The PCR-based identification was not possible due to its poor sequence quality, and therefore its full name is unknown. Based on microscopy and morphology alone and following the identification procedure according to Samson et al. (2004), one obtains the result *Penicillium camemberti*.

### 4.2.4 Super booster fruit powder

Table 10. *Result for super booster fruit powder, type of plating, weight of sample, name of fungi identified in microscope (PCR identification for 35 ºC), incubation temperature in* ºC *(inc. temp.), number of plates (contaminated/total) and size of the fungi in cm presented.*

| Product name | Type of plating | Weight | Inc. Temp. | Colour and size of fungi on primary isolation plates | Number of plates | Fungi identified |
|---|---|---|---|---|---|---|
| Super booster fruit powder | Direct | 0.3 g to 0.5 g | 25 ºC[b] | <1 cm. Olive green in the middle, dark blue outside of the middle and a small white margin. | 1/5 | *Aspergillus flavus* |
| | | | | 2-3 cm. Olive green with white margins that can be either large or small. | 2/5 | *Aspergillus nidulans* |
| | | | | 1-3 cm. Dark blue, with white spots. | 2/5 | *Aspergillus fumigatus* |
| | | | | 3-5 cm. Yellow brown with lighter margins. | 4/5 | *Byssochlamys spectabilis* |
| | | | 35 ºC[b] | 2-3 cm. Yellow brown with lighter margins. | 1/5 | *Byssochlamys spectabilis* |
| | | | | 2-3 cm. Olive green with white margins that can be either large or small. | 2/5 | *Aspergillus nidulans* |
| | | | | 1-2 cm. Dark olive green in the middle and large white margins, | 2/5 | *Aspergillus fumigatus* |
| | | | | 25 ºC: 3-4 cm. Blue with white margins. | | |
| | | | | 1-2 cm. Olive green in the middle and large white margins. | 2/5 | *Aspergillus flavus* |
| | 1:10 dilution | 1 g | 25 ºC[a] | <1 cm. Olive green in the middle, dark blue outside of the middle and a small white margin. | 1/2 | *Aspergillus fumigatus* |

[a] 0-3 colonies per plate, [b] 3-6 colonies per plate.

*Aspergillus* species were dominating all colonies. Most of the plates that showed any results of fungi had more than three colonies and most of them were merged

together, resulting in a restriction of colony sizes. None of the *Aspergillus* species had a microscopic structure of a biseriate head. The *Aspergillus* species growing in 25 °C incubated on direct plated plates, having yellow green and olive green colonies were determined to either be *A. parasiticus* or *A. flavus*. The only visible microscopic differences between the two is that *A. flavus* has a smoother stipe compared to *A. nidulans*.

*A. fumigatus* had a very typical dark blue colour with white margins, small with threadlike spots and microscopic details such as uniseriate and smooth stipe. All diluted plated plates showed a highly reduced number of colonies, resulting in one colony on one of the plates determined to be *A. fumigatus* due to its colony colour and shape.

Four of five plates incubated in 25 °C and one of five plates incubated in 35 °C had a fungus with a flat and powdery appearance and yellow and brown colour. It was determined by microscopy to be *Byssochlamys spectabilis* due to its microscopical structures such as rice shaped conidia, lacking columella, highly branched structure and that it has two to six conidiophores in chains. According to PCR identification it is supported to be *Byssochlamys spectabilis* (Houbraken et al., 2008; Samson et al., 2004).

### 4.2.5 Chia seed

Table 11. *Result for chia seed, type of plating, weight of sample, name of fungi identified in microscope (PCR identification for 35 °C), incubation temperature in °C (inc. temp.), number of plates (contaminated/total) and size of the fungi in cm presented.*

| Product name | Type of plating | Weight | Inc. Temp. | Colour and size of fungi on primary isolation plates | Number of plates | Fungi identified |
|---|---|---|---|---|---|---|
| Chia seed | 1:10 dilution | 10 g | 25 °C[d] | >8 cm, many colonies with colours ranging from white, black to blue and green. | 7/7 | - |
| | 1:10 dilution | 10 g | 35 °C[b] | 3-4 cm. White margins, black to brown middle with a white top. | 4/7 | *Aspergillus oryzae* |
| | | | | 25 °C: 3-4 cm. White margins, black to brown middle with a white top. | | |
| | | | | 4-6 cm. Light grey. | 2/7 | *Rhizopus stolonifer* |
| | | | | 25 °C: >8 cm. Dark grey with black margins. | | |
| | | | | 2-3 cm. Olive green in the middle with a white top, large white margins. | 4/7 | *Aspergillus flavus* |
| | | | | 25 °C: 4-5 cm. Small white margins and the rest is olive green. Have a small white middle. | | |
| | | | | 6-7 cm. Light grey. | 5/7 | *Lichtheimia ramosa* |
| | | | | 25 °C: >8 cm. White or light grey. | | |
| | | | | 6-8 cm. Dark grey. | 1/7 | *Rhizopus oryzae* |
| | | | | 25 °C: 3-4 cm. Dark grey/black, small white margins. | | |

[b] 3-6 colonies per plate, [d] more than 10 colonies per plate.

All plates with chia seeds showed growth indicating the product was heavily contaminated. None of the colonies could be identified on plates incubated in 25 °C due to all colonies being merged together and grown over each other, which made it difficult to isolate and identify them. The colonies grown in 35 °C were isolated and identified by both microscopy and PCR. However, a few of the colonies had an uncertain PCR result because of poor sequence quality.

*Aspergillus oryzae*, grown on four of seven plates, showed no difference in growth between 25 °C and 35 °C. It had a dark brownish middle and a fluffy appearance. In 25 °C, the middle part held small water droplets.

*Rhizopus stolonifer*, identified in two of seven plates and covering almost the whole plate, was identified by it microscopic structures such as large columella and apophysis, smooth stipe and no small branches. In 35 °C it was fluffy and

almost flat. It was shown to grow better at 25 °C, with a thick structure covering the whole plate up to the lid. This is supported by Samson et al. (2004), who state that its temperature optimum is 26 °C and the upper limit for growth is at 33 °C. The PCR result had poor sequence quality and could not confirm that the correct species identification was *R. stolonifer*, but supported that it was some kind of *Rhizopus* species.

*Rhizopus oryzae* could be found in one of seven plates and grew less at 25 °C than at 35 °C and had a flat and fluffy appearance, which is supported by Samson et al. (2004). The microscopic structures included large columella and apophysis, rough stipe and no small branches. The fungus covered the whole plate with a thick structure that reached the lid at 35 °C.

Three of seven plates contained *Aspergillus flavus* according to the PCR result. They all had a green layer with white margins, but also a white fluffy middle on top of the green layer. The green layer also had a powdery look. The microscopic structures showed a rough stipe with both bi- and uniseriate heads with a typical apical swelling.

*Lichtheimia ramosa* dominated the colonies and was found on five of seven plates. When studied in microscope it was suggested to be identified as *Lichtheimia corymbifera,* but PCR results showed that it was more probable to be *Lichtheimia ramosa.* Its microscopic structures showed small columella on small curved branches.

### 4.2.6 Probiotics

Table 12. *Result for probiotics Lacti plus and Probiotika life, type of plating, weight of sample, name of fungi identified in microscope (PCR identification for 35 ºC), incubation temperature in* ºC *(inc. temp.), number of plates (contaminated/total) and size of the fungi in cm presented.*

| Product name | Type of plating | Weight | Inc. Temp. | Colour and size of fungi on primary isolation plates | Number of plates | Fungi identified |
|---|---|---|---|---|---|---|
| Lacti Plus | Direct | 0,5 g | 35 ºC[a] | >8 cm. Dark grey and black with small white parts. | 1/5 | *Rhizopus oryzae* |
| | | | | 25 ºC: 4-6 cm. White bottom with a dark grey and black pigmented top. | | |
| | | | | 4-6 cm. White and grey. | 1/5 | *Mucor circinelloides* or *M. velutinosus* |
| | | | | 25 ºC: >8 cm. White. | | |
| | | | 25 ºC[a] | 4-6 cm. White bottom with a dark grey and black pigmented top. | 3/5 | *Rhizopus oryzae* |
| Probiotika life | Direct | 0,5 g | 35 ºC[a] | 4-6 cm. White. | 1/5 | *Geotrichum candidum* |
| | | | | 25 ºC: 3-4 cm. White and black with a grey middle. | | |

[a] 0-3 colonies per plate.

Most probiotic samples did not contain any fungal contamination, except for Lactic plus and Probiotika life, Lactic plus being the most heavily contaminated probiotic. *Rhizopus oryzae* was visible on one of five plates incubated in 35 °C and three of five plates incubated in 25 °C. This was supported by the PCR result and the textbook by Samson et al. (2004) when studied under microscope. It grew better at 35 °C than 25 °C and showed microscopic structures such as large columella, no small branches and almond shaped conidia.

One colony that could possibly be *Mucor circinelloides* or *M. velutinosus* could not be fully confirmed by the PCR result due to poor sequence quality giving 100 % targeting rate for both species. It showed microscopic structures such as small columella with simple and small bent branches and rough stipe. It grew better at 25 °C than in 35 °C and had a completely white and thick structure covering the whole plate.

The only colony found on a plate with incubated Probiotika life was determined to be *Geotrichum candidum* (with high uncertainty of identification). It was not possible to confirm its identity with PCR due to poor sequence quality. When studied under microscope it showed microscopic structures such as small branches, no columella and oval or almost square shaped conidia. The colony had a transparent, fluffy and almost flat appearance.

# 5 Discussion

## 5.1 Literature

*Aspergillus* spp. have among the mould opportunistic pathogens caused the majority of all IFIs, thus providing greater possibilities to study the most common species compared to other mould species of opportunistic pathogens also causing IFIs. The importance of studies of other species that cause IFIs, such as mucormycosis, increases due to a shown increasing trend of IFI cases caused by mucoromycetes. The increasing trend of detected IFI cases can possibly be an indication of better and more numerous analyses in the world. It could also be due to a larger world population, newer surgical trends and techniques with later occurring consequences or infections and diseases disrupting normal immune function. We are today better at dealing with organ failures and can do transplants, and for transplantation patients the risks for obtaining any infection increases highly, mostly due to the subsequent lifelong immunosuppressing medication (Kontoyiannis et al., 2010).

## 5.2 Product incubation and colony growth

The majority of the products had negative results in fungal growth (table 6), leaving eight of 16 (50 %) products containing any form of fungal contamination, and four of those were heavily contaminated. Most of the plates with fungal growth contained between one and six colonies after incubation. A few of the colonies did sometimes belong to the same species, but most of the time the colonies belonged to different species. Only the plates with direct plated chia seed and direct plated raw nettle leaf powder had too many colonies for the author to perform a complete isolation and identification. This difficulty was due to the fact that most of them were merged together. The diluted plating, on the other hand, gave results of between five and ten colonies per plate, and isolation and identification was possible to perform in these cases. On those plates that contained Zygomycetes it was harder to isolate other colonies due to the fast growth and size of most Zygomycetes species resulting in a plate full of thick mycelium with visible smaller and coloured colonies on the bottom of the plate.

This problem could occur on both non-diluted samples and diluted samples. Some of the colonies grown on camomile tea, morning leaf powder and super boosting food (table 7, 9 and 10) incubated at 25 °C were not fully identified with the help of sequencing due to the fact that they did not grow in 35 °C incubation. This could be due to inability to grow at 35 °C, i.e. they are not an opportunistic pathogenic species; alternatively, it could be a sampling effect which lead to their growth on 25 °C plates but not 35 °C plates. For example, *Aspergillus niger* sampled from the plate with diluted raw nettle leaf powder (table 8) incubated at 25 °C should also theoretically have been found in the plates incubated at 35 °C since *A. niger* also grows well at 35 °C (Samson et al., 2004). However, this was not the case. The sampling of plates incubated at 35 °C had one large colony of *Rhizopus microsporus* and several smaller colonies of *A. flavus* and *A. fumigatus*. The growth of these colonies could have restricted the growth of *A. niger*, explaining the absence of these. The plates with chia seeds (table 11) incubated in 25 °C did probably contain the species identified in table 11. *Rhizopus oryzae* on direct plated raw nettle leaf powder incubated at 35 ºC did most likely outgrow the other species that could be identified in table 8. Identification of *Byssochlamys spectabilis* growing on supper boost fruit powder (table 10) was challenging because it could easily be confused with *Paecilomyces variotii*; the anamorph of *Bussochlamys spectabilis*. This theory is supported by Samson et al. (2004) due to its similar powdery colony appearance, colour and microscopic structures (Houbraken et al., 2008).

## 5.3 Opportunistic pathogenic species in this study

All products with fungal growth had species that could grow in 35 °C except for the camomile tea. *Aspergillus fumigatus*, the most known opportunistic mould pathogenic species causing IFI, was identified in raw nettle leaf powder and super booster fruit powder. Several *Aspergillus* species were found in chia seed; one of them was *A. flavus. A. flavus* is another *Aspergillus* species commonly causing IFI, and it could be detected in all three products (raw nettle leaf powder, super booster fruit powder and chia seed). The last *Aspergillus* species detected was *A. nidulans* in super booster fruit powder and it has been reported as a dangerous pathogen to cause infections in lungs and bone, as well as entering wounds in the chest wall of immune compromised, hospitalized patients (Sophie et al., 2011).

In raw nettle leaf powder, colonies consisting of *Rhizopus oryzae* and *R. microsporus* could be found among the *Aspergillus* species. According to Pitt and Hocking (2009) *R. oryzae* causes zygomycoses and is according to De Hoog et al. (2000) known to mostly cause rhinocerebral infections. However, *R. microsporus* is known to mainly cause gastrointestinal infections, making this a high-risk species to cause IFI as a food-borne disease (De Hoog et al., 2000). The *R. microsporus* are not commonly found in food according to Pitt and Hocking (2009), but can be present in maize, black pepper, rice, soybeans and peanuts. In this study, *R. microsporus* was only found in raw nettle leaf powder originating from the northern Europe. *R. oryzae* was according to the results found in raw

nettle leaf powder, chia seeds and one of the probiotic samples. The last *Rhizopus* species detected was *R. stolonifer* in chia seeds, but no studies have found any reported cases of IFIs caused by *R. stolonifera*, as it cannot grow at body temperature. The upper limit of R. *stolonifer* is 33 °C and could be used as an indicator of that our incubation temperature was close to 32 to 33 °C instead of 35 °C, indicating on a false positive result. The probiotic product Lactic plus contained a *Mucor* species, either *M. circinelloides* or *M. velutinosus. M. circinelloides* is a known pathogen that infects both animals and humans, but no reports of gastrointestinal infections could be found (Pitt and Hocking, 2009).

In the morning leaf powder, *Lichtheimia ramosa* was the fungal species most commonly found, but this species was also found in chia seeds. *L. ramosa* is known to cause infections in the rhinocerebrum, pulmonary organs and damaged skin (Life-worldwide). These types of infections are increasing, especially among immune compromised hosts.

In the super booster fruit powder *Byssochlamys spectabilis* (*Paecilomyces variotii*) was found. It is a thermophilic species that can grow in 50 °C and is reported to cause mycosis, but rarely in the gastrointestinal tract (Samson et al., 2004; Pitt and Hocking 2009). This species has been reported to be found in peanuts, beverages and black peppers.

## 5.4 Identification in the industry

Identifying any contamination by the main opportunistic pathogenic fungal species in products produced in the industry is possible to do by an untrained person by following instructions for appearance of the colony and microscopical structures. This will not give a full accuracy in identifying the species, but most of the pathogenic species are possible to identify thanks to some of their basic colours and structures differentiating them from other species. *A. fumigatus* and *A. flavus* have their unique colour and colony shape for the *Aspergillus* species. *A.* oryzae can be confused with *A. flavus* due to their similarities, but is slightly darker in colour than *A. flavus*. *B. spectabilis* has powdery colonies, and both *R. microsporus* and *R. oryzae* are those species growing fast and usually cover the whole plate with a thick layer of mycelium.

It is also possible to reduce the number of non-pathogenic species by 35 °C incubation, which can be seen in table 7 and 8. This reduces the amount of unnecessary identification of species that are not opportunistic pathogens. It is however not possible to completely avoid fungal species not counted as pathogenic growing in 35 °C, which is the case in table 10 and 11.

## 5.5 Potential errors

Possible mistakes or errors are poor sequences that can result in a false positive result due to poor accuracy in matching species with the sequence given. Other

errors are potential contamination from the environment or in the air. All procedures were performed in a ventilated hood and all equipment were regularly sterilized with 70 % alcohol to avoid as much cross-contamination as possible. That no cross-contamination at all occurred cannot be fully guaranteed, but with the procedures followed in this study it is unlikely that any cross-contamination has occurred between products or from the environment or air. Other types of knowledge gaps in the study is contamination in other batches of the same product. Most of the products did not contain any conidial contamination, but it is possible that those batches were cleaner than others on the shelf in the store.

## 5.6 Future research

Number of replicates of direct plating per sample does not need to be increased, due to that most of the plates are very similar in number of colonies and species. In the future, diluted samples could be replicated to five plates per product and if containing more than three colonies per plate for x100 diluted samples, they can be further diluted to x1000. The number of samples of each product should also be increased to find if there could be any variation between batches of each product. There could also be an interest in studying other types of probiotic products, especially those marketed toward immune suppressed individuals.

To make it easier for the industry to work with fungal identification a study could be performed conducting fungal identification by using other types of medium that can isolate either *Aspergillus, Rhizopus, Lichtheimia* or *Fusarium* at 35 °C. These types of methods could give a more accurate result of pathogenic fungal species and make it easier to identify them because of fewer non-pathogenic species will be present to handle. However, using multiple selective media would increase the cost of every analysis.

# 6 Conclusion

Invasive fungal infection is not a reportable disease and can therefore hold a large number of unreported outcomes and causes. There is not enough data available to conclude how common gastrointestinal infections by fungi are and whether it was derived from food. There is also a knowledge gap about IFI caused by other species than *Aspergillus* and about the host's condition before the infection. Studies suggest that other factors negatively influencing the immune system might be a reason for an increased number of IFI cases. Another reason could be that more and newer surgical procedures take place.

This study could show that there is a possibility to isolate potentially pathogenic mould species in probiotic products marketed as immune boosting products. Other products marketed as supplements for people with weak immune systems did also contain several potentially pathogenic filamentous fungi species. All products containing fungi were dried and possibly crushed during processing, thus some of them were in a powdered form. It is possible that none of the contaminated products were heat-treated to reach a temperature which could destroy any fungal conidia. Probiotics are a product that should not be heat-treated because their base ingredients are live colonies of bacteria, and therefore it is an interesting choice of products due to that it can keep spores live inside and are a product marketed towards sensitive people. It was however not expected to obtain similar results for the dried nettle leaf powder, fruit powder or the super booster vegetable powder mixture as for the probiotics.

Incubating product samples at 35 °C instead of 25 °C will reduce the number of non-pathogenic species and give a slightly higher accuracy in growth of opportunistic pathogenic species. But this small reduction does not indicate that this method can fully work as a complete isolation technique for pathogenic filamentous fungi species. It can however simplify the identification process because of reduced growth of certain species.

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# Appendix

Project presented in popular science

## Potentially pathogenic fungi may infect the digestive tract of immune compromised people when eating contaminated food

Products marketed towards immune compromised individuals can actually be deadly for them. It is not the product itself that is dangerous, but the microorganism that may follow with it. A study has found that by screening several products marketed as supplements that are good for immune compromised individuals, several opportunistic pathogenic fungi species have been found. One of the most studied species called *Aspergillus fumigatus,* and another called *A. flavus* could be found in both powdered leaf and fruit products. Both of them are common pathogens known to be infecting wounds and lungs and in some cases the digestive tract of immune compromised people. The spores of these fungi can usually be found in the environment surrounding us, both indoor and outdoor. However, when eating contaminated food there is a risk of having an infection in the digestive tract with a mortality rate of 85 %, which is much higher than if infected in for example the lungs. One of the results from the study showed that *Rhizopus microsporus* could be found in one of the powdered leaf products. This is worrying because *R. microsporus* is commonly infecting the digestive tract. Several probiotic products were screened in this study and the results showed that one of them contained the opportunistic pathogenic species *R. oryzae.*

Eight of 16 products could show signs of fungi growth. These products have probably not been heat-treated to a temperature that can destroy the spores. Surprisingly, only two of eight probiotic products could show growth of fungi. These products were expected to have higher numbers of fungi spores because they are not pasteurized. Instead a high level of safety procedures is evident in the industry.

### Further reading


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