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Micromycetes contaminating nuts used for food

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Nuts are one of the most delicious and nutritious foods, but due to the extremely high fat, protein and low water content they are quite refractory to spoilage by microorganisms. In this study a wide range of microorganisms was recorded. The samples were collected from 6 different markets in Kaunas, Lithuania. The moisture content of nuts was determined by drying the samples in an oven at 103 (±2) °C temperature for 6 hours. Dilution plating (a surface-spread method) was used for colony counting. The analyses of nut samples revealed that imported nuts were quite intensely contaminated with propagules of various fungi species. The moisture content in the nuts varied in dependence of their type and acquisition place. The highest moisture content was observed in walnuts, 13.93%, while almost twice less in pistachio and hazelnuts, 6.38 and 6.76%, respectively. The least number of colony forming units (cfu) of fungi was detected in hazelnuts (9.11×10^3 cfu/g) followed by peanuts (11.46×10^3 cfu/g). In contrast, walnuts (20.90×10^3 cfu/g) represented the highest infections of fungi. A total of 8 different fungal genera (*Aspergillus* spp. *Acremonium* spp., *Cladosporium* spp., *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Paecilomyces* spp., and *Penicillium* spp.) and 16 species were isolated. *Aspergillus*, *Mucor* and *Penicillium* genera were more frequently detected than other genera of fungi. *Aspergillus* spp. was found in the most investigated nut samples. The greatest diversity of micromycetes was detected in hazelnuts and walnuts.

Key words: *Aspergilus,* hazelnuts, moisture content, peanuts, pistachio, walnuts

INTRODUCTION

Nuts are globally recognized as a nutritionally important food, being high in proteins, vitamins and minerals such as potassium, iron, vitamin E, thiamine, niacin, riboflavin and unsaturated fats. Various nuts are used as a raw material in many industries as well as for direct consumption (Abdulla, 2013).

During maturation in the field, nuts are exposed to a variety of environmental conditions that increase the risk of fungal contamination.

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The outer shell of nuts affords natural protection against the most contaminating agents such as insects and microorganisms, making the nutmeat virtually microbe-free (International Commission on Microbiological Specifications for Foods, 1998). After the nuts are harvested, appropriate conditions and materials are necessary to protect against spoilage during storage ant transport. High temperatures, humid conditions and extreme temperature gradients during storage and transport can increase the susceptibility of nuts to spoilage (International Commission on Microbiological Specifications for Foods, 1998). Nuts require cool and dry conditions to maintain their low moisture content. After postharvest used drying technologies nuts contain approximately 8% moisture and have an approximate water activity of 0.7. This low moisture content minimizes spoilage concerns due to bacteria. However, this along with low-soluble carbohydrate levels and hygroscopic properties creates a favourable environment for the survival of moulds (Compendium of the Microbiological Spoilage of Foods and Beverages, 2009). Due to extremely high fat and protein of various nuts products are quite refractory to spoilage by microorganisms. Mould can grow upon them if they are stored under conditions that permit sufficient moisture for their propagation (Phillips et al., 1979; Frank, 1981). Processing is also a critical factor in controlling nut spoilage. Shelling and other processing such as chopping and slicing can increase contamination of nutmeat with fungi.

Many of dried nuts are favourable for the storage moulds such as *Eurotium* spp., *Aspergillus* spp., *Penicillium* spp., and *Wallemia* spp. Hazelnuts are most susceptible to *Rhizopus* spp., *Penicillium* spp., *Aspegillus* spp. contamination during harvest, postharvest handling and storage. In freshly harvested pistachios *Fusarium* (particularly *F. equiseti* and *F. acuminatum*) and *Alternaria* species are also important components of the postharvest microbiota (Hocking, Pitt, 1996). The intimate contact of soil with the shells of developing peanuts is an ideal situation for fungal colonisation. *Aspergillus* species, particulary *A. niger* and *A. flavus*, have been reported frequently. Previous studies showed that 30.97 million tons of greasy seed products, mainly pistachio and peanut, of different Asian and African countries were contaminated by *A. flavus* and *A. parasiticus* (Christensen, Meronuck, 1986; Dekoe et al., 2000; D'Mello, Macdonald, 1998). Other commonly occurring fungi in peanuts are *Fusarium* (*F. solani, F. semitectum, F. oxysporum*), *Penicillium* (*P. funiculosum*, *P. chrysogenum*, *P. citrinum*, *P. aurantiogriseum*), *Rhizopus* (*R. oryzae*, *R. stolonifer*), *Rhizoctonia solani*, *Macrophomina phaseolina*, *Chaetomium* and *Culvularia* species (King et al., 1981; Pitt et al., 1992; Hocking, Pitt, 1996).

Mould contamination in the nuts is prevalent and mycotoxin contamination is a significant food safety issue due to serious adverse effects on human health (Raudonienė, Lugauskas, 2005). A major problem related to fungal attack in nuts is the production of toxic secondary metabolites, particularly fumonisin, zearalenone and aflatoxin, produced by *F. verticillioides, F. graminearum* and *A. flavus*, respectively (Scott, 1993). Aflatoxin has powerful teratogenic, mutagenic and hepato-carcinogenic effects (Wang et al., 2001). Aflatoxins were detected in 90% of hazelnut samples at levels of 25–175 μg kg^{-1} and in 75% of wallnut samples. Mycotoxins can cause toxic effects on human and animal tissue and organs. They are among the 21st century major concerns due to their important role (Jay et al., 2005).

The aim of the study was to evaluate the spread of micromycetes and assess a risk related with consumption of nuts supplied in the market. Even though some of these fungi are not toxigenic, contamination with different *Aspergillus, Fusarium* and *Penicillium* spp. should be monitored for maintenance of foodstuff hygiene and safety.

MATERIALS AND METHODS

Unshelled hazelnuts (*Corylus* sp.), peanuts (*Arachis* sp.), pistachio (*Pistacia vera*), walnuts (*Juglans* sp.) were collected from 6 different markets in Kaunas, Lithuania. Moisture content of nuts was determined by drying the samples in an oven at 103 (±2) °C temperature for 6 hours.

The moisture content of the sample was calculated as percentage (%) difference between the sample before and after drying (Pitt et al., 1992). Dilution plating (a surface-spread method) (Pitt, Hocking, 1997) was used for colony counting. 10 g of each milled nut sample were homogenized with 90 ml sterile water for 30 min in an orbital shaker. Serial decimal dilutions up to 10^{-4} were made and 0.1 ml aliquots were inoculated in triplicate onto the Petri dishes with the Sabouraud glucose agar medium with chloramfenicol (0.5 g l^{-1}). The dishes were kept in a thermostat at the temperature of 26 (±2) °C. Growing colonies of fungi were counted on 3rd, 5th and 7th day. Fungi were identified according to morphological and microscopic characteristics (Pitt, Hocking, 1999; Watanabe, 2002; Leslie, Summerell, 2006).

Analyses were performed in five replications.

RESULTS AND DISCUSSION

The analyses of nut samples revealed that imported nuts were quite intensely contaminated with propagules of various fungi species. The moisture content in the nuts varied in dependence of their type and acquisition place. Storing conditions had the effect on the moisture content of nuts, not only their chemical composition. The highest moisture content was observed in walnuts, 13.93%, while almost two times less in pistachio and hazelnuts, 6.38 and 6.76%, respectively (Table 1). The highest fluctuations in the moisture content between the same nut samples were in walnuts – from 11.28 to 17.08%.

The least number of colony forming units of fungi was detected in hazelnuts (9.11×10^3 cfu/g) followed by peanuts (11.46×10^3 cfu/g). In contrast, walnuts represented the highest infections of fungi (20.90×10^3 cfu/g) (Table 1). This contamination could be due to long-term storage, marketing under non-hygienic conditions of the food products in the poor environmental conditions including high moisture and temperature.

The results presented in Table 2 show the identity of fungi that was found in all nut samples. A total of 8 different fungal genera (*Aspergillus* spp., *Acremonium* spp., *Cladosporium* spp., *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Paecilomyces* spp., and *Penicillium* spp.) and 16 species were isolated (Table 2).

Table 1. Nut moisture content and contamination with micromycetes

Samples	Hazelnuts	Walnuts	Pistachio	Peanuts
Moisture content, %				
1	5.62 A c	11.28 D a	6.50 B c	8.06 C b
2	7.34 C b	17.08 D f	6.02 A a	7.06 B a
3	5.92 A d	16.60 C e	5.94 A a	7.04 B a
4	6.74 B a	14.74 D c	6.22 A b	11.14 C e
5	7.78 B f	16.38 D d	6.96 A e	8.98 C d
6	6.30 A e	12.02 D b	6.62 B d	8.26 C c
Average	6.76 A	13.93 C	6.38 A	8.56 B
Contamination (colony forming units), g × 10^3				
1	6.56 A a	19.76 D b	12.58 C d	10.16 B a
2	6.88 A ab	22.98 D d	11.52 C c	10.20 B a
3	6.88 A ab	20.98 D a	10.50 C a	9.04 B b
4	7.38 A c	20.94 D a	10.76 B a	15.00 C e
5	13.60 B d	21.22 D a	14.96 C b	12.06 A c
6	7.14 A bc	21.92 D c	14.94 C b	12.94 B d
Average	9.11 A	20.90 D	12.54 C	11.46 B

Significant differences ($p < 0.05$) among the nuts in rows are marked by different capital letters A, B, C, D.

Significant differences ($p < 0.05$) among the markets in the column are marked by different lowercase letters a, b, c, d

The greatest diversity of micromycetes was detected in hazelnuts and walnuts.

Table 2. The isolated micromycetes from nuts

Samples	Fungi
Hazelnuts	*Aspergillus fumigatus*
	A. niger
	A. versicolor
	Fusarium sprotrichioides
	Penicillium chrysogenum
	Penicillium spp.
	Rhizopus stolonifer
Walnuts	*Aspergillus niger*
	Aspergillus spp.
	Cladosporium spp.
	Mucor spp.
	Penicillium chrysogenum
	P. crustosum
	P. expansum
	Penicillium spp.
Pistachio	*Acremonium* spp.
	Aspergillus flavus
	A. niger
	A. fumigatus
	Penicillium spp.
Peanuts	*Aspergillus flavus*
	A. fumigatus
	Aspergillus spp.
	Mucor spp.
	Paecilomyces variotii

Aspergillus, *Mucor* and *Penicillium* genera were more frequently detected than other genera of fungi. *Aspergillus* spp. was found in the most investigated nut samples.

Frank (1981) detected about 50 fungal species in walnuts: the most commonly occurring ones were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, and *Cladosporium* sp.

Black or brown species of *aspergilli* were found in 90% of the investigated samples and *A. niger* was the most frequently isolated mold in walnuts, contaminating 80% of walnut samples (Figure). Species of *Penicillium* dominated in all of the walnut samples, and in many cases the plates of the lowest dilution were totally covered with *Penicillium*. *P. crustosum* was identified in 60% of the samples (Figure). *P. expansum* was potentially found in 50% of the samples and assessed as dominating. Potential isolates of *P. chrysogenum* were found in 45% of samples.

Aspergillus species appear to be the most frequent reported fungi from pistachios. Doster, Michailides (1994) and Heperkan et al., (1994) reported 14 *Aspergillus* species in pistachios (*A. flavus, A. parasiticus, A. niger, A. ochraceus* and others).

In the analysed pistachios and peanuts the most prevalent fungi were *Aspergillus* spp. (identified in 70% of the samples), and *Penicillium* spp. (40%) was the predominant mould. Peanut samples were contaminated by *Aspergillus flavus*. Isolated fungi were the following: *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor*. The previous study revealed that the presence of *Penicillium* and *Aspergillus* in soil may be the main causes of the contaminations in peanuts. Regarding direct contact of the soil with the peanuts in growth phases, fungi can penetrate through the peanut's shell and grow there (Pitt et al., 1991). *A. niger* and *A. flavus* showed similarities where both species were found in all pistachio samples. Species of the genera *Aspergillus* and *Penicillium* occurred most frequently in the pistachio samples.

Abdel-Hafez and Saber (1993) reported the following fungi in hazelnuts: *Aspergillus* (*A. flavus*, *A. fumigatus*), *Penicillium* (*P. aurantiogriseum, P. chrysogenum, P. citrinum, P. oxalicum*), *Cladosporium* (*C. cladosporioides, C. herbarum*), *Fusarium* (*F. equiseti*, *F. moniliforme*, *F. oxysporum*), *Rhizopus stolonifer*, *Rhizomucor pussilus*.

Penicillium spp. was the predominant genus isolated from all investigated hazelnut samples. Also frequent prevalent fungi in hazelnuts were *Aspergillus fumigatus*, *A. flavus*, *A. versicolor*, and *Penicillium chrysogenum*. Some hazelnuts were contaminated by *Fusarium sprotrichioides* (Figure).

The frequency of isolation of the mycotoxigenic fungi may be actual danger. They possess the property of releasing toxins into the food products. *A. fumigatus* belongs to *Aspergillus* genus and produces such toxins as fumigaclavines, fumigalin, fumigatin and others. *Aspergillus*

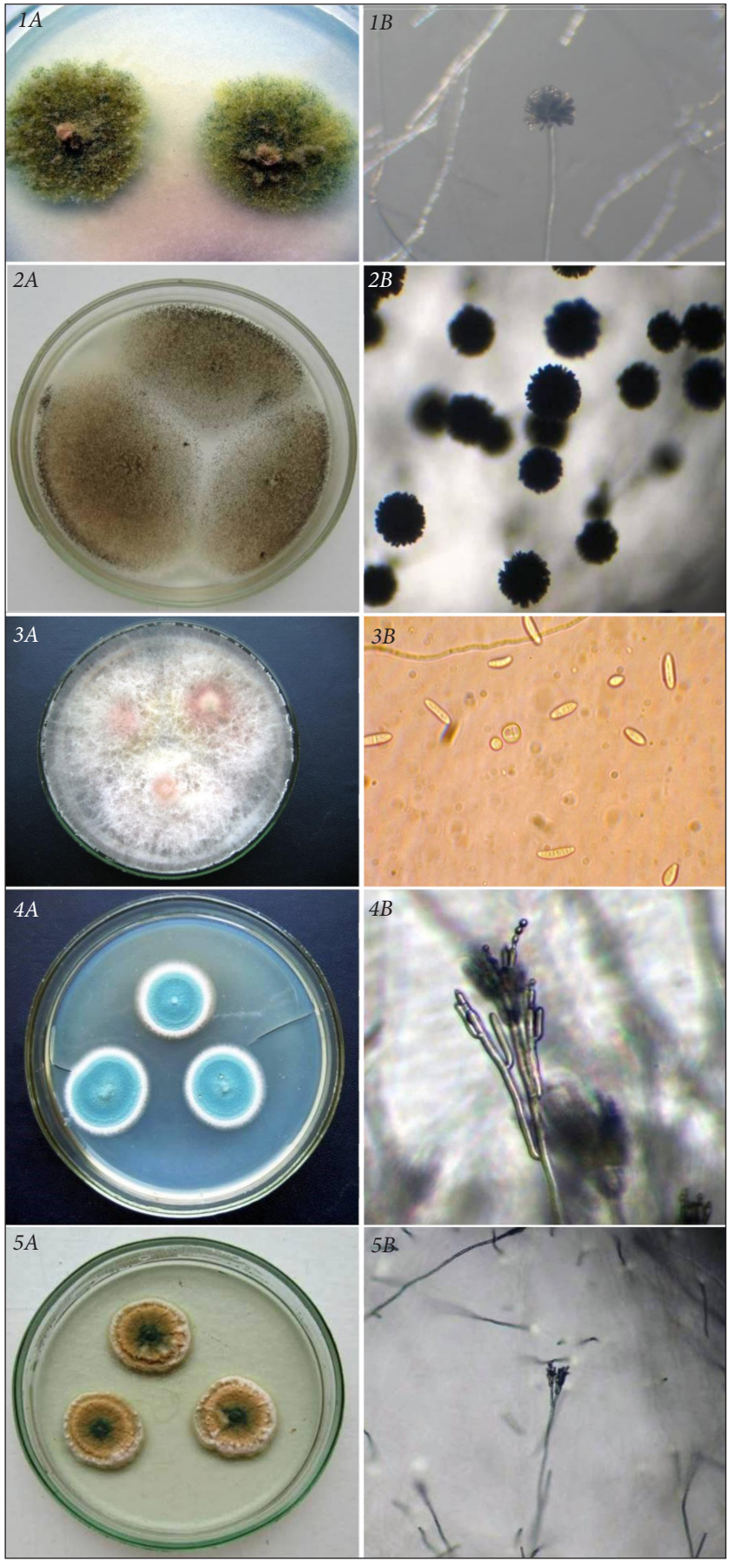

Figure. Fungi colonies (A) and conidia (B) on Sabouraud glucose agar:
1 – Aspergillus flavus;
2 – Aspergillus niger;
3 – Fusarium sprotrichioides;
4 – Penicillium expansum;
5 – Penicillium chrysogenum

flavus produces secondary metabolites aflatoxins that have powerful teratogenic, mutagenic and hepato-carcinogenic effects. About 10% of hazelnut samples were contaminated by *Fusarium sporotrichioides* which is a producer of trichothecenes and zearalenone. Some species of the *Penicillium* genus actively producing toxins are *P. chrysogenum* that produces roquefortine C, PR-toxin, xantocillin and *P. expansum* which is able to produce patulin and citrinin.

CONCLUSIONS

The results of this study showed that nut contamination with fungi was prevalent especially in high moisture content nuts. Storing conditions of nuts have effects on moisture content and contamination with fungi in the same nut type:

1. The highest moisture content was observed in walnuts, 13.93%, while almost twice less in pistachio and hazelnuts, 6.38 and 6.76%, respectively.

2. The least number of colony forming units of fungi was detected in hazelnuts (9.11 × 103 cfu/g) followed by peanuts (11.46 × 103 cfu/g). Walnuts represented the highest infections of fungi (20.90 × 103 cfu/g). The greatest diversity of micromycete species was detected in hazelnuts and walnuts.

3. A total of 8 different fungal genera (*Aspergillus* spp. *Acremonium* spp., *Cladosporium* spp., *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Paecilomyces* spp., and *Penicillium* spp.) and 16 species were isolated from nuts. *Aspergillus* spp. was found in the most investigated nut samples.

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RIEŠUTŲ UŽKRĖSTUMAS MIKROMICETAIS

Santrauka

Riešutai yra vienas labiausiai vartotojų mėgstamų maistingų produktų, kurių sudėtyje yra daug riebalų ir baltymų. Dėl juose esančio mažo drėgmės kiekio jie nėra gera terpė mikroorganizmų vystymuisi, todėl yra atsparūs mikrobiologiniam gedimui. Tirti šešiose skirtingose Kauno prekybos vietose įsigyti keturių rūšių riešutų mėginiai. Drėgmės kiekis riešutuose nustatytas mėginius džiovinant 103 (±2) °C temperatūroje šešias valandas. Siekiant nustatyti mikromicetų kolonijų skaičių, skiediniai buvo pasėti į mitybinę terpę. Mikrobiologinė analizė atskleidė, kad importuoti riešutai yra gausiai užteršti įvairių genčių mikromicetais. Drėgmės kiekis riešutuose priklauso nuo jų rūšies ir įsigijimo vietos. Didžiausias drėgmės kiekis nustatytas graikiniuose riešutuose – 13,93 %, beveik perpus mažesnis pistacijų ir lazdynų riešutuose – atitinkamai 6,38 ir 6,76 %. Mažiausias mikromicetų pradų skaičius nustatytas lazdynų riešutuose – 9,11 × 103 bei žemės riešutuose – 11,46 × 103 ksv/g. Didžiausia mikrobiologine tarša išsiskyrė graikiniai riešutai, čia bendras mikromicetų pradų skaičius sudarė 20,90 × 103 ksv/g.

Riešutuose rastos aštuonios mikromicetų gentys (*Aspergillus* spp., *Acremonium* spp., *Cladosporium* spp., *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Paecilomyces* spp., *Penicillium* spp.) ir išskirta šešiolika jų rūšių. Didžiausia mikromicetų įvairovė nustatyta lazdynų ir graikiniuose riešutuose. *Aspergillus*, *Mucor* ir *Penicillium* genčių mikromicetai vyrauja visuose riešutų mėginiuose, tačiau dominavo *Aspergillus* genties mikromiceai.

Raktažodžiai: *Aspergilus*, drėgmės kiekis, graikiniai, lazdynų riešutai, pistacijos, žemės riešutai