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MOLECULAR DETECTION OF FUNGI IN PAPRIKA, CHILI POWDER AND BLACK PEPPER

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Abstract

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Paprika powder, chili powder, and black pepper are among the most frequently used spices in the world. The Internal Transcribed Spacer (ITS) regions were identified by high sequence similarity with the ITS regions of many microscopic fungi, especially representatives of the phylum *Ascomycota*, from 18 different spice samples that were examined. However, supplied quality certificates indicated that 10 of the 18 samples had no contamination present and were safe for human consumption. The various genera of fungi that were identified from the spices are considered to be a food safety concern as they are able to produce mycotoxins. Qualitative detection was supplemented by positive detection of viable fungal DNA using qPCR for the genera *Aspergillus*/*Penicillium* in two paprika powder and black pepper samples. These results concurred with the control analysis using axenic cultures. The described methods can be used for routine testing of spices to provide safe spices and other products to consumers.

Keywords: contamination, DNA analysis, identification, spices

INTRODUCTION

Paprika (*Capsicum* L., Solanaceae) and black pepper (*Piper nigrum* L., Piperaceae) are two of the most commonly used spices throughout the world with a high economic importance (Surh, 2002; George *et al*., 2017). Black pepper and paprika are commonly used as a dried powder to season food without any further processing or cooking (Sardiñas *et al*., 2011). However, black pepper and paprika powder can cause serious health problems due to microbial contamination (Tulu *et al*., 2014). The critical processes that have been identified as likely routes of spice contamination are improper agronomic treatments, inappropriate storage, and lack of proper sanitary conditions from the vendor. Inadequate quality of the spices may affect consumer health, thus continuous education concerning the safe production and storage of spices is crucial to guarantee high-quality spices are available to consumers (Keit, 2009; Tulu *et al*., 2014).

Microbial pathogens are one of the most frequent contaminants of dried spices. The origin of the contaminants may be from different sources, such as the indigenous microflora of the plant and growing environment, post-harvest contamination, irrigation with contaminated water during production, microorganisms present in processing facilities, and from contact with people who handle the spices during production. An overall decline in the number of microbial pathogens present is observed following washing and processing of the spices, however, the remaining microogranisms are usually aerobic spore-forming bacteria and

common molds (Ahene *et al.*, 2011). Some fungi (e.g. *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium* and *Stachybotrys*) are capable of mycotoxins production which can cause illness to people and animals if they are consumed (Santos *et al.*, 2010; Zain, 2011; Hammami *et al.*, 2014). In addition, the conditions in which plants for paprika, chili powder and black pepper production are grown are often characterized by high temperatures and humidity that can promote the growth of various microorganisms (Garbowska *et al.*, 2015). Several studies have demonstrated that spices have an overall high levels of microbial contamination in various spices. This has led to a need to identify the microbial contamination present and to identify ways of preventing it from occurring (Aiko and Metha, 2016; Banach *et al.*, 2016; Reinholds *et al.*, 2017). *Bacillus*, *Aspergillus*, *Penicillium* and *Micrococcus* have been reported in dried spices from different parts of the world (Mckee, 1995; Garcia *et al.*, 2001; Banerjee and Sarkar, 2003). Delcoure *et al.* (1994) analyzed pepper powders for contamination by microorganisms. The mold flora was dominated by xerophilic species, particularly *Aspergillus fumigatus, A. flavus, A. niger*, and *A. ochraceus.* Species of *Aspergillus*, *Penicillium* and *Rhizopus* were generally contaminants of all analyzed samples of red powder peppers in Turkey (Erdogan, 2004).

The detection and enumeration of microorganisms in food and food contact surfaces are an important component of any integrated program to ensure food safety throughout the supply chain (López-Campos *et al.*, 2012). Different methods, like Howard's test, specific colorants, cultivation techniques, immune- and physicochemical methods have been used for detection and identification of microorganisms in food (Bisha and Brehm-Stecher, 2010; Jasson *et al.*, 2010). The axenic cultures, which show typical growth and sporulation, are used for the identification of different types of filamentous fungi (Hutchinson, 1991). However, reliable and unambiguous identification by morphological characteristics is demanding due to variations within individual families. Accurate identification requires a high level of expertise and this is the main reason why DNA-based methods are increasingly used for identification. These methods have many benefits such as faster and more reliable detection. In addition, DNA-based methods have high specificity and sensitivity compared to conventional cultivation methods. Fast and precise determination of potentially dangerous microorganisms in foods is essential to guarantee safe and high-quality products for consumers (Luque *et al.*, 2012). New microbial detection methods in foods are based on the detection of DNA polymorphisms (e.g. DNA microarray and sequence analysis), which enables detection of multiple pathogens and gives qualitative and quantitative information at once (Mandal *et al.*, 2011). Propidium monoazid (PMA) is fluorescent photo-affinity labels that covalently binds to nucleic acid after photoactivation, but only is able to enter cells with compromised cell walls/membranes. The DNA that is covalently bound to this dye cannot be amplified using PCR. This method has been used for the identification of live vs. dead bacteria cells (Nocker *et al.*, 2006), viable and non-viable conidia of species of infectious fungi (Vesper *et al.*, 2008), and for determining viable wine yeast (Andorrà *et al.*, 2010).

DNA barcodes have been developed to identify variability in individual species by specific DNA regions (Hebert *et al.*, 2003). ITS (Internal Transcribed Spacer) of rDNA, the most important DNA regions in microorganisms, plants and animal species, were examined (Schocha *et al.*, 2012). Specific and selective primers for the ITS region for specific detection of the fungal DNA from DNA mixtures obtained from different matrices were proposed by Gardes and Bruns (1993). ITS sequences were used for identification and comparing of genetic diversity of different ascomycete genera members (mainly *Penicillium*, *Fusarium* and *Aspergillus*) in different localities in China (Wu *et al.*, 2013). Accurate identification should then be followed by quantification which is possible by using axenic cultures (Tranquillini *et al.*, 2017) or qPCR (quantitative PCR) (Luchi *et al.*, 2005).

The aim of this study was to assess the possibility of molecular methods for the qualitative and quantitative detection of contaminations in samples of paprika, chili powder, and black pepper. Identification of the microbial contaminants can provide insight into the sources of contamination and ways to prevent contamination from occurring.

MATERIALS AND METHODS

DNA identification of fungal contaminants from spices

A total of 15 samples of hot and sweet paprika powder, two samples of black pepper and one sample of chili powder (TRUMF International, Prerov, Czech Republic) were used for this study (Tab. I). The PCR reaction mixture had a total volume of 25 µl and contained 0.5 U *Taq* polymerase (Promega, Madison, WI, USA), 1 × aliquot Green Go *Taq*® reaction buffer, 0.1 mmol/l of each deoxynucleotide (Promega), 0.3 mol/l of each primer (ITS1-F 5' – CTTGGTCATTTAGAGGAAGTAA-3' (Gardes and Bruns, 1993) and ITS4 5' – TCCTCCGCTTATTGATATGC-3' (White *et al.*, 1990) and 20 ng of template DNA. The conditions for PCR using a T3 thermocycler (Biometra, Götingen, Germany) were as follows: 3 min incubation at 94 °C, then 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and the cycle repeated 40 times. The reaction was held at 72 °C for 10 min before a final hold at 10 °C. DNA isolation, sequence analysis of ITS region, and bioinformatic analysis were performed according to the protocol described by Vyhnánek *et al.* (2017).

Detection of viable fungal DNA

For detection of viable fungal DNA, ground spice samples 3, 10, 12 and 18 (Tab. I) were used. The samples were selected due to detection of *Aspergillus* and/or *Penicilium*. Fungal DNA detection was carried out using 50 mg of paprika and 100 mg of pepper. The samples were mixed in 500 µl of phosphate buffered saline (PBS) buffer. One half of the samples were placed in a thermoblock and heated to 90 °C (Biosan Bio TDB-100) for 20 min, placed on ice for 2 min, and then mixed. All samples were centrifuged at 13,000 *g* for 5 min, and the supernatant was removed. A total of 500 µl elution buffer (EB) and 10 µl of 2.5 mM PMA reagent (part of BLU-V Viability PMA Kit, Qiagen) was added. The samples were incubated in the dark at room temperature for 10 min. After incubation, all of the samples were placed in the Blu-V System (Qiagen, Hilden, Germany) and irradiated for 15 min with mixing every 2 min. All samples were then centrifuged at 13,000 *g* for 5 min, the supernatant removed, and 400 µl AP1 buffer from the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) was added to the pellet (Qiagen, 2015). The DNA concentration and purity was determined using a Picopet 1.0 (Picodrop, Cambridge, United Kingdom). Detection of fungal DNA was performed using qPCR (Bio-rad CFX 96, USA) under the following conditions: initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 seconds, annealing and elongation at 60 °C for 2 min. This was repeated for a total of 45 cycles. The mastermix for qPCR was used with a total volume of 25 µl: 12.5 µl Microbial qPCR Mastermix, 1 µl Microbial DNA qPCR Assay, 5 µl DNA sample (3.2 ng/µl for paprika and 5.8 ng/µl for pepper) and 6.5 µl Nuclease free water (Microbial DNA qPCR Assay Kit Pan *Aspergillus/Penicillium* Qiagen, USA). The analyses were carried out in two biological and three technical repetitions. Based on the comparison of the Ct (cycle of threshold) values in the heat-treated and non-heat treated samples, it was possible to identify the viable DNA from the *Aspergillus* and *Penicillium* species.

Identification of fungal contaminants from axenic cultures

Dichloran-Glycerol agar (DG-18; Neogen Europe, Scotland, UK), Oatmeal Agar (OMA; Merck, Germany); Czapek yeast autolysate medium (CAY agar; HiMedia Laboratories, India) and Potato-dextrose agar (PDA; Neogen Europe, Scotland, UK) were used to obtain axenic cultures of fungi from paprika powder (3 and 12) and black pepper (10 and 18; Tab. I).

The chosen samples of paprika (10 µg of paprika powder) and pepper (10 pieces) were placed on Petri dishes (90 mm) containing PDA or DG-18. All samples were plated in triplicate and incubated for 7 days at 25 °C with a 12/12 light dark cycle. The various fungi were examined visually and/or by using a stereomicroscope with different

I: *Samples of spices used for DNA analysis*

No.	Name	Country of origin	Moisture content (%)+
used in production (certificate of quality)			
1.	sweet paprika powder	Hungary	7.05
2.	sweet paprika powder	Hungary	8.61
3.	sweet paprika powder	Hungary	9.33
4.	sweet paprika powder	Hungary	9.51
5.	hot paprika powder	Hungary	8.94
6.	sweet paprika powder	Hungary	8.66
7.	paprika powder	Hungary	8.54
8.	paprika powder	Hungary	7.99
9.	black pepper	Vietnam	12.4
10.	black pepper	India	12.0
unused in production (without certificate of quality)			
11.	hot paprika powder	Hungary	8.69
12.	sweet paprika powder	Hungary	9.10
13.	sweet paprika powder	Hungary	8.45
14.	hot paprika powder	Hungary	9.05
15.	paprika powder	Hungary	8.88
16.	paprika powder	Hungary	8.75
17.	chili powder	Hungary	8.86
18.	black pepper	Vietnam	12.2

+ – characteristics supplied by TRUMF International, Ltd. Prerov, Czech Republic

magnifications (Olympus BX12 and Olympus BX 41, Olympus Europa Holding GMBH Hamburg, Germany). All colonies showing different colors were subcultured separately on OMA. Daily observations were made to fungal colonies that were present on the plates. The fungi were isolated and pure cultures were prepared from a single spore (Choi *et al.*, 1999) for identification.

Aspergillus was revived on OMA and *Penicillium* on CYA and incubated at 25 °C for 7 days. Colony characteristics of *Aspergillus* and *Penicillium* were recorded and morphological characteristics were observed on a slide in 3% lactic acid. The diameter of the colonies was recorded after incubation for 4 days and re-incubation for 7 days. The taxonomic identification of fungi (based on macroscopic and microscopic characteristics) was performed using previously described methods (Rapper and Fennel, 1977; Pitt, 1991; Singhn *et al.*, 1991; Samson *et al.*, 2014; Visagie *et al.*, 2014). Colonies from the four clones of axenic cultures was used for DNA isolation, PCR amplification and amplicon purification via the same protocols as described above except using IT4 primer for direct sequencing by Macrogen. Final sequences were evaluated using NCBI database and the BLAST algorithms (blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

A total of 18 spice samples (14 of sweet and hot paprika powder, 3 of black pepper, and 1 of chili powder), were divided into two groups according to the final processing and were analyzed by PCR and sequence analysis of ITS1 and ITS2 region using

II: *Fungi identified from spice samples determined by sequence analysis*

No.	Genus	Number of sequences	GenBank Accession Number
1	*Alternaria*$^{+}$, *Cladosporium*, *Fusarium*$^{+}$, *Galactomyces*, *Geotrichum*, and *Pichia**$^{+}$	8	MF155966 to MF155973
2	*Botryotinia*, *Botrytis*, *Candida*$^{+}$, *Cladosporium*, *Cryptococcus**, *Galactomyces*, *Geotrichum*, *Pichia**$^{+}$, *Sclerotinia*, *Sporobolomyces* and *Symmetrospora*	8	MF155974 to MF155981
3	*Aspergillus*$^{+}$, *Botryotinia*, *Botrytis*, *Candida*$^{+}$, *Eurotium*$^{+}$, *Galactomyces*, *Penicillium*$^{+}$, *Pichia**$^{+}$, *Sclerotinia* and *Tilletia*	8	MF155982 to MF155989
4	*Botrytis*, *Botryotinia*, *Galactomyces*, *Geotrichum*, *Malassezia*$^{+}$, *Pichia**$^{+}$ and *Sclerotinia*,	8	MF155990 to MF155997
5	*Candida*$^{+}$, *Cryptococcus*, *Galactomyces*, *Geotrichum* and *Pichia**$^{+}$	7	MF155998 to MF156004
6	*Cladosporium*, *Cryptococcus*, *Galactomyces*, *Geotrichum* and *Pichia**$^{+}$	8	MF156005 to MF156012
7	*Alternaria*$^{+}$, *Penicillium*$^{+}$ and *Scopulariopsis*	9	MF156013 to MF156021
8	*Alternaria*$^{+}$, *Cladosporium* and *Cryptococcus**	9	MF156022 to MF156030
9	*Beauveria*$^{+}$, *Bjerkandera*, *Candida*$^{+}$, *Colletotrichum*, *Cryptococcus**, *Ganoderma*, *Issatchenkia*$^{+}$, *Malassezia*$^{+}$ and *Thanatephorus*	10	MF156031 to MF156040
10	*Aspergillus*$^{+}$, *Cyberlindnera*, *Pichia**$^{+}$ and *Wallemia*$^{+}$	4	MF156041 to MF156044
11	*Alternaria*$^{+}$, *Colletotrichum*, *Diaporthe*, *Glomerella*, *Phomopsis* and *Xeromyces*	9	MF156045 to MF156053
12	*Alternaria*$^{+}$, *Aspergillus*$^{+}$, *Cladosporium*, *Galactomyces* and *Eurotium*$^{+}$	12	MF156054 to MF156065
13	*Alternaria*$^{+}$, *Fusarium*$^{+}$, *Hanseniaspora*, *Lewia*, *Pichia**$^{+}$, *Pseudozyma* and *Moesziomyces*	12	MF156066 to MF156077
14	*Alternaria*$^{+}$, *Botryotinia*, *Botrytis*, *Galactomyces*, *Geotrichum*, *Gibberella*, *Fusarium*$^{+}$ and *Sclerotinia*	11	MF156078 to MF156088
15	*Alternaria*$^{+}$, *Botryotinia*, *Botrytis*, *Cryptococcus**, *Galactomyces*, *Penicillium*$^{+}$ and *Sclerotinia*	12	MF156089 to MF156100
16	*Alternaria*$^{+}$, *Botryotinia*, *Botrytis*, *Cladosporium*, *Galactomyces*, *Geotrichum* and *Pichia**$^{+}$	10	MF156101 to MF156110
17	*Cryptococcus**, *Colletotrichum*, *Gibellulopsis*, *Glomerella* and *Verticillium*	12	MF156111 to MF156122
18	*Aspergillus*$^{+}$, *Colletotrichum* and *Eurotium*$^{+}$	9	MF156123 to MF156131

* – yeast; $^{+}$ – potential mycotoxin producers

primers ITS1-F and ITS4. Primer ITS1-F is specific for fungal organisms so only the fungal ITS regions were separately amplified from total DNA mixture.

Altogether, 166 sequences were obtained (in average 9.22 sequence per sample) and analyzed using the NCBI database and the BLAST algorithm (Tab. II). From the samples that had certificates of purity and lack of contamination, 79 sequences were obtained (No. 1–10), while the remaining 87 sequences were detected in samples without the certificate and were never introduced into food manufacturing. However, these sequences were recorded in the NCBI database and were assigned a GenBank accession number (MF155999–MF156131). For all sequences, except for MF156042, a 99 to 100% match with the known fungal taxon in the NCBI database was found. The MF156042 sequence had an 87% match with a *Wallemia* genus representative. A total of 13 samples matched with three yeast genera (*Candida*, *Cryptococcus* and *Pichia*). Genera that are able to produce mycotoxins were identified in all but one sample (No. 17–chili powder). In the remaining samples, the presence of *Alternaria*, *Beauveria*, *Candida*, *Eurotium*, *Fusarium*, *Issatchenkia*, *Malassezia*, *Penicillium*, *Pichia* and *Wallemia* (all capable of mycotoxin production) were detected.

Based on the fungi and yeast life cycle, an estimation of the possible mode of contamination of spices was determined (Table III). The time of contamination was divided into the categories as follows: during vegetation, storage, processing, and unable to be determined. Based on our findings, the most frequent occurrence of contamination was observed during vegetation, where 32 genera of fungi and yeast were detected. The lowest number of spice contamination was recorded during the processing phase (7 genera).

III: *Fungal contaminates identified from spice samples and possible contamination sources*

During vegetation	During storage	During processing	Unable to determine
Alternatia	*Alternaria*	*Aspergillus*	*Alternaria*
Aspergillus	*Aspergillus*	*Cladosporium*	*Botrytis*
Beauveria	*Botrytis*	*Malassezia*	*Bjerkandera*
Botryotinia	*Candida*	*Penicillium*	*Cryptoccocus*
Botrytis	*Cladosporium*	*Pichia*	*Diaporte*
Candida	*Cyberlindnera*	*Wallemia*	*Ganoderma*
Cladosporium	*Eurotium*	*Xeromyces*	*Pichia*
Colletotrichum	*Fusarium*		*Phomopsis*
Cryptococcus	*Gibberella*		*Symmetrospora*
Cyberlindnera	*Lewia*		*Tilletia*
Eurotium	*Malassezia*		
Fusarium	*Penicillium*		
Galactomyces	*Pichia*		
Geotrichum	*Xeromyces*		
Gibellulopsis			
Glomerella			
Hanseniaspora			
Issatchenkia			
Lewia			
Malassezia			
Moesziomyces			
Penicillium			
Pichia			
Pseudozyma			
Sclerotinia			
Scopulariopsis			
Sporobolomyces			
Thanatephorus			
Tilletia			
Verticillium			
Wallemia			
Xeromyces			

The identification of fungi and yeasts in the spice samples was done through detection of viable genomic DNA of *Aspergillus*/*Penicillium* genera using the PMA and qPCR protocols. In addition, the detection of the presence of genomic DNA from two samples with (one of paprika powder sample No. 3 and one of black pepper No. 10) and two without (paprika powder No. 12 and black pepper No. 18) a guarantee of contamination-free certificate were chosen (Fig. 1). The differences in Ct values of the heat treated and non-heat treated samples shows that a higher content of viable DNA of *Aspergillus* and *Penicillium* genera was in the paprika powder sample No. 12 where the Ct value was 4.38 compared to sample No. 3 (1.20) for which health and food safety insurance certificate was issued. However, in both samples of black pepper, it was observed that the difference was proportional between Ct value of the destroyed and non-destroyed samples (No. 10–1.48 and No. 18–1.43). It can be concluded that there were no differences between the samples with and without the certificate since the differences in the amount of viable fungi DNA were not observed.

After verification of the sequence amplification from representatives of two genera, namely *Aspergillus* and *Penicillium*, further cultivation and identification of fungi were performed using the axenic culture of the same samples that were used for qPCR. The ITS region from the pure cultures were then sequenced and analyzed using the NCBI database and the BLAST algorithm (Table IV). Based on the microscopic evaluation of the axenic cultures, representatives of the *Penicillium* genera were identified in samples with a certificate that the samples were free of contamination (paprika powder No. 3 and black pepper No. 10). In samples without a certificate, *Aspergillus* genera were identified from paprika (sample No. 12), while with black pepper (sample No. 18) representatives of both genera (*Aspergillus* and *Penicillium*) were detected. These results were confirmed by sequencing the ITS regions of the pure fungi acquired from axenic cultures, where a total of 7 sequences (average 1.75 per sample) were obtained with 100% match with *Aspergillus* and *Penicillium* genus. These sequences were recorded in the NCBI database and GenBank accession number (MF156132–MF156138) was assigned.

DISCUSSION

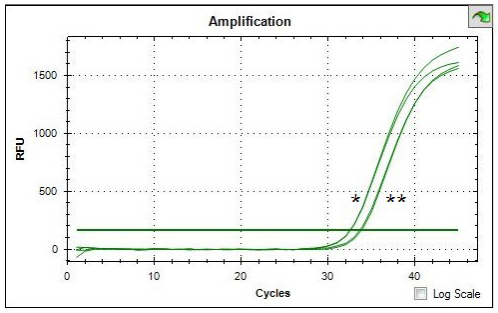
The most important contaminants of spices, from a food safety perspective, are mycotoxins, pesticide residues and toxic (heavy) metals (Reinholds *et al.*, 2017). However, de Nijs *et al.* (2017) expanded this list of contaminants for spices to include plant toxins. Plant toxins are defined as secondary metabolites which are produced by many plants and are not essential for the survival of the plants itself, but are toxic to human health. DNA barcoding methods are increasingly used in recent years for the identification of microorganisms in foods (Hebert *et al.*, 2003). In the case of fungi, the ITS sequence is primarily used for this purpose (Xu, 2016). ITS sequences appear suitable for identification of fungal species, as well as for trace amounts of fungi in the sample due to high numbers of ITS region copies in the genome compared to other methods. In the study of Wu *et al.* (2013), 177 species of *Penicillium*, *Fusarium*, *Aspergillus*, *Trichoderma* and *Talaromyces* were identified based on ITS in soil samples collected at 20 different localities in China. For fungi species identification, beside the well-known GenBank database, specialized databases such as Mycobank (www.mycobank.org) databases can be used for comparison. Mycobank databases contains more than 50,000 ascomycete sequences. In our study, we focused on the possibility of using DNA analysis of the ITS sequence for the identification of contamination in 18 spice samples (paprika and chili powder, black pepper) that had either the presence or absence of a certificate of a produce free of contamination from an accredited laboratory. Similar conclusions were described by Schocha *et al.* (2012) and Tedersoo and Lindahl (2016). A part of this study was focused on testing the use of the qPCR method for identifying DNA from fungi that are viable. All sequences (166) which were detected in this study for the ITS region were recorded in the NCBI database and Genbank accession numbers were assigned. In 72.2% of analyzed samples, a consensus sequence representative of yeast (3 genera), were systematically classified into the taxonimic group of *Ascomycota* by Weiss *et al.* (2013).

The biggest problem of fungal food contamination is that some species have potential to produce mycotoxins that can have significant adverse effects on human health (Hammami *et al.*, 2014). Aiko and Mehta (2016) found that the most problematic genera of toxigenic fungi for medicinal plants and spices in India are representatives of *Aspergillus*, *Penicillium* and *Fusarium*. Moreover, these genera were also detected in Brazilian black pepper, white pepper and Brazil nuts (Freire *et al.*, 2000), in fresh sweet peppers and their processed products (Gambacorta *et al.*, 2018) and Capsicum powder (Santos *et al.*, 2011). In our study, mycotoxin producers were identified in each sample except chili powder (No. 17). In addition, we have identified another 8 genera (*Alternaria*, *Beauveria*, *Candida*, *Eurotium*, *Issatchenkia*, *Malassezia*, *Pichia* and *Wallemia*) in the remaining samples, which can be considered as mycotoxin producers (Ismaiel and Papenbrock, 2015). Representatives of *Aspergillus* can produce specific mycotoxins, i. e. aflatoxins (Rezaei *et al.*, 2017), while *Penicillium* is able to produce both citrinin and ochratoxin (Ismaiel and Papenbrock, 2015). Taxon *Fusarium* is able to produce several mycotoxins, of which fumosin, deoxynivalenol and zearalenone are the most important due to their negative impact on human and animal health (Shephard, 2011). Representatives of *Alternaria* are able to produce tenuazonic acid, alternariol, and alternariol methyl ether (Van de Perre *et al.*, 2015).

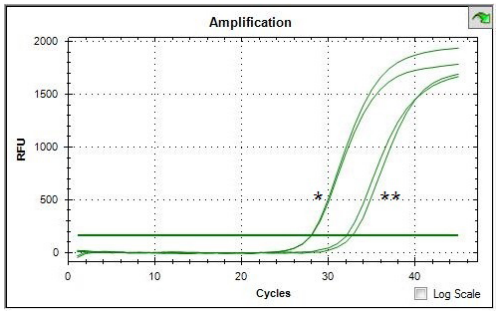
Molecular identification of mycotoxins producers can increase efficiency and reduce the cost of the quantification of individual mycotoxins by molecular methods, e.g. ELISA (enzyme-linked immunosorbent assay) or HPLC (high-performance liquid chromatography) (Urusov *et al.*, 2015; Chen *et al.*, 2016). Most food processes have variable effects on mycotoxins reduction. In general, most thermal treatments used in food production reduced mycotoxins concentration significantly, but do not eliminate them completely (Milani and Maleki, 2014).

To ensure safe food production, it is necessary to monitor the occurrence of pathogenic organisms including fungi during different stages such as growing, harvesting, storing and processing of raw materials into the final product. Hashem and Alamri (2010) divide fungi into two ecological categories, e.g., field and storage fungi. Field fungi were observed to invade developing or mature seeds while it is on the plant. The major field fungal genera are *Alternaria*, *Fusarium* and *Cladosporium*. Storage fungi are those encountered on plants at moisture conditions routinely found in stored products. These fungi are principally species of *Aspergillus* and *Penicillium* (Abou Donia, 2008). Spices can be contaminated with fungi mainly during spice processing, storage and transport (Dimić *et al.*, 2008). In our study, we divided the identified fungal genera into four categories. Based on the results obtained (number of identified genera), it is clear that contamination is most likely to occur during growing until harvest. This means that only a high quality starting material is essential for the production of safe spices.

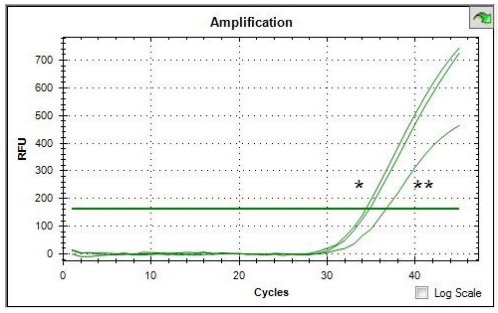
Many methods can be used for the diagnosis of pathogenic fungi: axenic cultures with classification according to fungi morphology, serological analysis and molecular diagnostic methods (Kozel and Wickes, 2014). Axenic cultures are used as



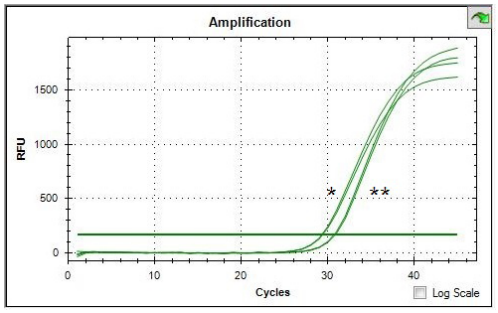
A



B



C



D

E

Spice	No.	Ct value (average ± SD)		
		Heat treated	Non-heat treated	Negative control (ultrapure water)
Paprika powder	3	32.51 ± 0.02	33.72 ± 0.05	44.01 ± 0.02
	12	28.04 ± 0.02	32.42 ± 0.10	43.59 ± 0.03
Black pepper	10	34.64 ± 0.08	36.12 ± 0.01	43.89 ± 0.05
	18	29.33 ± 0.02	30.76 ± 0.05	44.08 ± 0.04

1: *Comparison of Ct values of DNA viable and non-viable cells by qPCR*

Legend: A – paprika powder (No.3), B–paprika powder (No.12), C–pepper (No.10), D–pepper (No.18), E–comparison of mean Ct values with negative control, *–non-heat treated variant, **–heat treated variant. RFU–relative fluorescence units, SD–standard deviation. Differences between the Ct values of the heat and non-heat treated samples are presented.

IV: *Fungal contaminates identified from axenic cultures*

Spice	No.	Axenic culture	DNA analysis	
			Genus	GenBank Accession Number
Paprika powder	3	*Penicillium*	*Penicillium olsonii*	MF156132
	12	*Aspergillus*	*Aspergillus penicillioides*	MF156138
Black pepper	10	*Penicillium*	*Penicillium olsonii*	MF156133 to MF156135
	18	*Penicillium* and *Aspergillus*	*Penicillium olsonii* and *Aspergillus penicillioides*	MF156137 and MF156138

a standard for quantification and are an integral part of many legislative standards around the world (Banach *et al.*, 2016). In our study, axenic cultures were successfully applied to verify fungal genera detected by molecular diagnostic methods. In addition, de Nijs *et al.* (2017) determined that analysis of the ITS region is an appropriate method for fungal identification. Based on the variability in the ITS region, we have identified 36 genera of fungi and yeast. The disadvantage of this method is that it is unable to quantify the DNA from viable fungi and yeasts. However, it is possible to use another molecular diagnostic method, qPCR, coupled with a PMA treatment prior to PCR, to distinguish and quantify DNA from living cells of the microorganisms from the dead cells (Nocker *et al.*, 2006; Vesper *et al.*, 2008). In our study, we successfully used this method to detect viable DNA of *Aspergillus*/*Penicillium* genera representatives in samples of paprika powder and black pepper. Our results confirmed the successful application of PCR for the detection and quantification of *Aspergillus* (Goebes *et al.*, 2007; Suleman and Somai, 2012) and *Penicillium* (Arquiza and Hunter, 2014; Tannous *et al.*, 2015) species in agriculture and the food industry.

The original hypothesis that samples without certificate of guarantee that the product is free of contamination will contain a higher amount of viable DNA was found only in paprika powder, where the difference between the Ct of heat treated and non-heat treated samples was about 4 times greater than the samples with a certificate. For greater application of the method and accurate quantification of viable DNA using qPCR, it will be necessary to create calibration curves for the most significant fungal genera so that it can be compared in practice with the quantification expressed by colony-forming unit per gram (CFU/g). However, despite this disadvantage Elizaquível *et al.* (2014) recommends this method due to its specificity and sensitivity of the procedure, combined with its speed, its reliability, and the potential automation of the technique. However, this method has several advantages to routine analysis programs that assess the presence and viability of fungi in food commodities.

CONCLUSION

Contamination of spices (paprika and chili powder, black pepper) may occur during different stages of the production process. However, it was observed that the most frequent occurrence of contamination was during growing. This is important if some of the fungal genera responsible for contamination are potential mycotoxin producers (e.g. *Aspergillus*, *Fusarium* or *Penicillium*). Secondary metabolites of fungi, such as mycotoxins, are an important parameter in terms of health and safety monitoring of spice production and other foods for humans, but also for animal fodder. Moreover, the increasing use of molecular techniques used to identify microbial contaminates in foods is continuing. The optimization of these methods can lead to the formation of automated and rapid detection tests that can also be applied to enforce legislative standards.

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