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Incidence of fungal contamination in a Romanian bakery: a molecular approach

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Abstract

Fungal infections are responsible for the quality and safety of bakery products. The present approach aims to screen the incidence of fungi species in a Romanian bakery and to identify the Aspergillus strains that produce aflatoxins.

Conventional methods were used to isolate the fungal strains from the air in different bakery areas, and molecular techniques (multiplex PCR reaction, ITS-RFLP method) were employed for the selective detection of A. flavus, A. niger, A. fumigatus, and A. ochraceus.

The results obtained by conventional methods revealed that fungal contamination was different from one area to another, the smallest contamination being detected in the fermentation room, 2.5 cfu/m^3, and the highest in the partition of dough (14 cfu/m^3) and in the slicing and packing (14 cfu/m^3) areas. The most frequent genera in all production areas, identified by the colonies morphology and microscopic examination were: Aspergillus, Penicillium, Alternaria, Fusarium, Ulocladium, Neurospora and Trichoderma. The presence of Neurospora genus fungi was also noticed in some specific areas, like the toast and packaging area, the cooling area and package storage room; however, the counting of colonies was not possible.

Molecular techniques, performed on isolated Aspergillus strains (the most frequently recorded), revealed that results were highly correlated with classical identification, with the exception of two samples of A. fumigatus and one sample of A.ochraceus, suspected to be contaminated.

Although classical methods based on fluorescence appearance on the coconut medium did not show the presence of A. flavus aflatoxin producing strains, the simultaneous presence of both PCR products corresponding to nor1 and aflpR genes suggests that at least one strain of the isolated A. flavus could produce aflatoxin.

Keywords: bakery fungal contamination, Aspergillus, multiplex PCR reaction, ITS-RFLP method, aflatoxins

Introduction

Many problems in bakery products are produced by fungal infections. The growth of these microorganisms during production and storage of the products are especially important for different genera: *Alternaria, Aspergillus, Botrytis, Candida, Cladosporium, Penicillium* and *Saccharomyces* (*14*). Microbial spoilage is a problem because it can induce nutritional losses, off-flavors, formation of mycotoxins and potentially allergenic spores. Therefore, besides being an economic problem, unwanted fungal growth can cause some serious health hazards that have to be monitored carefully. The fungal contamination in bakeries is due to natural contaminants of grains and flour, as well as to spores in airborne dust (*5*). Among fungal species, those belonging to *Aspergillus* genus are of interest because some of them

were reported as fungal pathogens and as aflatoxin producers and others have been exploited for biotechnologically interesting products (*4, 7*). Generally, identification of *Aspergillus* species is based on the morphological characteristics of the colony and microscopic examination, and depends on the experience of the examiner (*7*). It is generally accepted that morphological analysis is time consuming and requires isolation and purification of each fungal species prior to identification (*17*). Several studies dedicated to molecular methods used for the identification of *Aspergillus* species and other spoilage fungi showed that ITS - PCR followed by sequencing the amplification products may be a useful method for the identification of the fungal species (*6, 8,13*) and nested PCR using a mixture of specific primers can lead to identification of *Aspergillus* species (*7*). Recently primer sets were designated that allow the identification of some *Aspergillus* species from DNA isolated directly from different sources, without purification of fungal cultures (*17, 10*).

Aflatoxins are potent carcinogenic and mutagenic secondary metabolites produced by several *Aspergillus* species. Their presence in food and feed commodities, alone or in association with other mycotoxins (ochratoxin, DON etc) is of particular importance due to their potentially harmful effects on humans and livestock. For *Aspergillus* species, extensive biochemical and genetic studies have been also performed to better understand the aflatoxin biosynthesis and the molecular regulation of the process. It was reported that the aflatoxin biosynthesis involves 23 enzymatic reactions for conversion of acetyl-coenzyme A to aflatoxin B1, and 25 genes, clustered in a 75 kb DNA region, are responsible for coding the proteins of this pathway. Among these genes, *aflR, nor1, apa2, omt1* or *ver I* are designated as targets for PCR detection systems (*16*).

This work aimed at screening several production areas of a Romanian bakery for fungal contamination, by conventional and molecular methods and to evaluate the mycotoxin producing potential of several *Aspergillus* isolates.

Materials and methods

Isolation of fungal strains

For isolation of contaminant fungi, Petri dishes with glucose cloramphenicol agar (Biokar Diagnostic) were exposed to air for 15 min, in different areas of the bakery. The colonies formed at the surface of solid nutrient medium were counted as an average on 5 Petri dishes and results were calculated based on Omelianski method (*11*).

Spores from each representative fungal colony were taken, suspended in 1 ml of sterile distilled water containing 0.1% Tween 20 and subjected to dilution plate technique on PDA agar to isolate pure cultures. Plates were incubated at 25^{o}C for 5-7 days and the isolated colonies were examined for their morphological appearance, smell and light microscopy morphology.

DNA extraction from fungi

Two approaches for DNA isolation were used. Firstly, DNA was isolated from pure cultures: mycelium from each of 18 fungal colonies belonging to *Aspergillus* genus was obtained by culturing for 4 days at 27^{o}C in GYEP broth (2 % glucose, 0.3 % yeast extract, 1 % peptone). Genomic DNA was isolated using the method described by Kumar and Shukla (*9*). In the second approach, each Petri dish containing contaminant fungi from different production areas were scrapped to recover mycelium and conidia and incubated for 12 h in 4 ml of absolute ethanol (Sigma) at 22^{o}C, according to Richard et al. (*17*). Mycelium and conidia were then placed into a mortar with quartz sand and mechanically disrupted. All the subsequent steps of the isolation technique were the same as described by Kumar and Shukla

(*9*). DNA was recovered after isopropanol precipitation and resuspended in 50 µl of TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0).

PCR primers and PCR amplification conditions

PCR was used to amplify target fragments specific for *Aspergillus flavus, A.ochraceus, A.niger* and *A.fumigatus*, as well as for detecting the potential of some fungal strains to produce aflatoxins. The primer sequences used in our experiments are listed in table 1. All the primers were obtained from HVD Life Sciences.

Table 1. Primers used for identification and characterization of fungal isolates

Primer	Sequence	Target	References
APA-450	TATCTCCCCCCGGGCATCTCCCGG	Regulatory gene *aflR*	*8*
APA-1482	CCGTCAGACAGCCACTGGACACGG		
nor1	ACCGCTACGCCGGCACTCTCGGCAC	Structural gene *nor1*	
nor2	GTTGGCCGCCAGCTTCGACACTCCG		
ITS1	TCCGTAGGTGAACCTGCGG	18S rDNA universal fungal 5' primer	*6, 20*
ITS3	GCA TCG ATG AAG AAC GCA GC	5.8S rDNA universal fungal 5' primer	
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rDNA universal fungal 3' primer	
AFMF76	TAC CTG CAA ACC GGA TTA CG	*A. fumigatus*	*7*
AFMR129	TTT GCT GCC GAA CTG GCT CGA C		
ANGF79	CAC GTT CAA GCC GGA ACT ACG C	*A. niger*	
ANGR139	CAA GAT GTT GTC CAT CAC CGC T		
AFOF28	CAC ATT CAA GCC AGA TTA CG	*A. flavus*	
AFOR95	GCT TAG GGT TGT TCA TAC GAG CAC		
OCRA1	CTT CCT TAG GGG TGG CAC AGC	*A. ochraceus*	*15*
OCRA2	GTT GCT TTT CAG CGT CGG CC		

.Every ITS-PCR reaction contained 12.5 µl template DNA, both forward and reverse primer at 0.20 mM, each dNTP at 0.10 mM, 10 µl 10*x* PCR buffer and 2.5 U Taq DNA polymerase in a final reaction volume of 100 µl. An initial denaturation step at 94^{o}C for 5 min was followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 56^{o}C for 45 sec and extension at 72^{o}C for 1 min, with a final extension step of 72^{o}C for 7 min. Restriction analysis was performed by using Hha I enzyme, according to the manufacturer (Promega).

Multiplex PCR assays were performed in 25 µl of a reaction mixture that contained: 2.5 µl buffer, 1.5 µl $MgCl_2$, 0.5 µl dNTPs, 0.5 µl Taq polymerase (Fermentas), 0.5 µl primer *nor1* (Reverse), 0.5 µl primer *nor1* (Forward), 0.5 µl primer *aflR* (reverse), 0.5 µl primer *aflR* (Forward), 1.5 µl template DNA, and 16.5 µl deionized water that was added to make the volume up to 25 µl. PCR amplification conditions were 5 min for denaturation at 96°C, followed by 35 cycles: 95°C, 1 min; 65°C, 30 s; 72°C, 30 s for the first cycle; and 94°C, 30 s; variable (56 - 62°C), 30 s; 72°C, 30 s for the next 34 cycles.

Amplified products were visualized by 1.5 % agarose gel electrophoresis in TBE buffer (0.09 M tris, 0.09 M boric acid and 2 mM EDTA, pH 8.3), stained with ethidium bromide, and photographed.

Aflatoxin production. The potential of aflatoxin production was examined on coconut agar medium for several *Aspergillus* isolates using the method described by Davis et al. (*3*).

Results and discussion

Isolation and identification of fungal contaminants

Foods prepared in traditional and modern industrial bakeries are vulnerable to microbial contamination due to the nature of the raw material used and to standard handling practices for bakery products. The fungi collected from air samples from different places of the bakery emphasized that the fungal contamination is not uniform. The smallest contamination was detected in the fermentation room (2.5 cfu/m^3), while the highest was detected in the areas dedicated to partition of dough (14 cfu/m^3) and in the bread slicing and packing area (14 cfu/m^3) (table 2).

Table 2. The frequency of fungal spores in different bakery areas

Air sampling areas	Fungi, CFU/m^3
Kneading room	6.5
Dough partition area	14
Fermentation room	2.5
Pre-cooling area	8.1
Cooling room	4.6
Bread slicing and packing area	14
Passage hall	5.7
Package storage room	4.9

A high diversity of fungal colonies was detected, grown at the surface of the solid medium. The most frequent genera, in all production areas, identified by the colonies morphology and microscopic examination were: *Aspergillus, Penicillium, Alternaria, Fusarium, Ulocladium, Neurospora* and *Trichoderma.* Of the fungal isolates, the strains of *Aspergillus* with 16.8 % occurrence were most frequently recorded, followed by *Penicillium* (14.5 %), *Alternaria* (14.9 %), *Trichoderma* sp. (3.6 %), *Ulocladium sp.* (7.8 %) and *Fusarium* (3.2 %) (fig. 1). Strains of *Rhizopus, Mucor* or *Mortierella* were detected to a lesser extent (<7.5 % all), in samples collected from some areas.

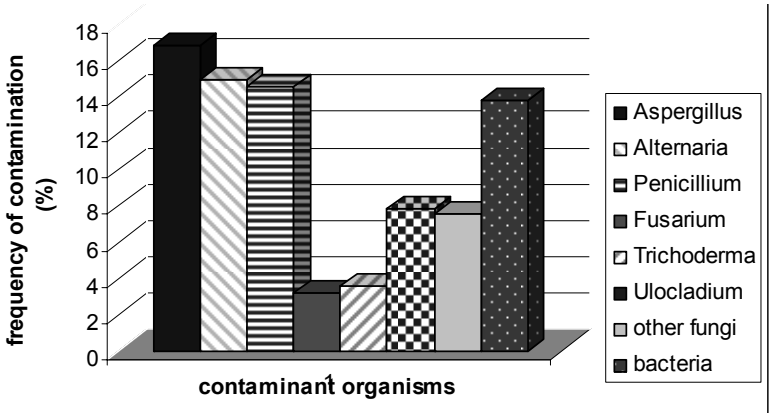

Figure 1. Air-borne fungal contamination in bakeries

In addition to these fungi, in some specific areas, such as the toast and packaging area, cooling area and package storage room, the predominant genus was *Neurospora*, but the counting of the colonies was impossible.

Moreover, differences between production areas for the contamination with aspergili and other fungal species were observed. In the kneading room, the contamination and the diversity of fungal species was reduced, the dominant fungi being aspergili (>90%). In the

fermentation room, the contamination is reduced (as average number of contaminants) but the diversity was increased (table 3).

Table 3. Contaminants in the bakery production areas

Contaminant	Kneading room	Pre-cooling area	Cooling room	Dough partition area	Fermentation room	Bread slicing and packing area	Passage hall	Package storage room
Aspergillus	92.85%	26.45%	0	0	29%	18.7%	19.6%	5.3%
Penicillium	0	14.2%	13.6%	0.8%	13%	16.6%	29.9%	17.05%
Alternaria	0	28.4%	2.3%	25.75%	8%	10,35%	10.3%	0
Trichoderma	0	0	0	0	0	11.4%	0	8.51%
Fusarium	0	12.25%	1.4%	3%	1.6%	0	4.12%	0
Ulocladium	0	8.4%	0	0	0	0	2.1%	58.5%
Mortierella	7,15%	0	0	0	0	0	0	0
Neurospora	0	0	80/7%	0	0	40.2%	0	0
Mucor	0	0	0	0	0	2.75%	0	0
Rhizopus	0	0	0	0.8%	24.2%	0	0	0
Other fungi	0	0	0	0	6.5%	0	0	8.52%
Bacteria	0	0	0	69.7%	17.7%	0	6.2%	2.12%

The *Aspergillus* species isolated from different bakery areas were identified primarily by conventional techniques. Based on cultural and morphological characteristics, several species were distinguished: *A.candidus, A.flavus, A.ochraceus, A.niger, A.fumigatus, A.repens* and *A.wentii* (fig. 2).

Figure 2. Diversity of the contaminant fungi isolated from bakery

Among the aspergilli isolated from bakery, the occurrence of *A. niger* (32.45 %) was the highest, followed by *A. ochraceus* (19.28 %), *A.candidus* (17.85 %), *A.repens* (17.14 %), *A.flavus* (7.8 %), *A.wentii* (4.47 %) and *A. fumigatus* (1.01 %). The detection of such variety of *Aspergillus* species in production areas, as well as the presence of *Fusarium* in several areas suggests a possible presence of mycotoxins (aflatoxins and/or DON) that could contaminate bakery products.

Molecular identification of fungal species

Many species of fungi are pathogenic and toxigenic. Thus, identification of potentially harmful microbes to species level is important for early diagnosis and environmental monitoring.

It is known that *Aspergillus* is a large genus composed of more than 180 accepted anamorphic species, with teleomorph described in nine different genera, which are finally divided into Sections. In order to establish a molecular method for rapid diagnosis of the contamination with a certain *Aspergillus* species, it is necessary to find the best combination of primers that could allow, in one reaction, the discrimination among several aspergili. For this purpose, some strains of *Aspergillus* were examined, which were isolated from the bakery for their response, at DNA level, to different primers.

DNA isolated from 18 strains of *Aspergillus* was used as template and PCR was carried out with two combinations of primer pairs: ITS3/ITS4 that amplifies the 5,8S-28S rDNA region, and ITS1/ITS4 that amplify the 18S - 28S region. When primer pair ITS3/ITS4 was used, a unique amplicon of approximately 350 bp in size was obtained for all tested aspergili. No significant differences in amplicon size were noted among the various *Aspergillus* species tested. The results suggest a good conservation, at least as size, of 5,8S - 28S rDNA region, similar aspects being presented by other researchers (*1*). With the primer pair ITS1/ITS4, an amplicon of approximately 600 bp was obtained for all five strains considered as *A. niger* and for those of *A. flavus,* which is in accordance with the data from literature (12). Similar amplicons, as size, were obtained for *A. ochraceus* strains, but different in the strains considered as *A. candidus*. The treatment of the amplicons with HhaI restriction enzyme led to the observation of clear differences among species. The results obtained for *A. flavus* and for *A. niger* showed that the sizes of restriction fragments produced by HhaI restriction enzyme are similar to those reported by Mirhendi et al. (*12*): 89, 143, 179, 184 bp for *A. flavus,* and 90, 124, 179, 207 bp for *A. niger*, respectively.

The specificity of the primers for the identification of *Aspergillus* species was evaluated by PCR, using genomic DNA isolated from various strains isolated in pure cultures, and identified by microscopic analysis and colonies characteristics. Several strains designated as *A.flavus* were tested with the primer AFOF28/AFOR95 described by Kanbe et al. (*7*), based on DNA polymerase II gene from *A.flavus*. Specific primers derived from the same gene (from corresponding species) were proposed by the same authors for *A.niger* (primer pair ANGF79/ANGR139) and *A.fumigatus* (AFMF76/ AFMR129). The amplicons obtained had the predicted size for 2 strains considered as *A. flavus* (679 bp) and *A. niger* (600 bp), respectively (fig.3). For other four isolates designated also as *A.flavus,* the size of the amplicon was slightly shorter: 650bp. None of the suspected colonies to be *A.fumigatus* produced the specific amplicon.

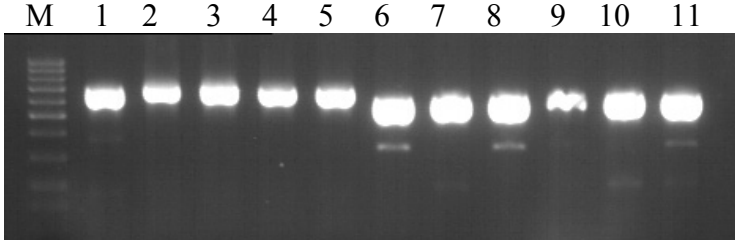

Figure 3. Agarose gel analysis of multiplex PCR products obtained from different *Aspergillus* strains. M = 100bp DNA ladder (Roth); lanes 1 – 5 = *Aspergillus spp.*; lanes 6 - 11 = *A.niger*.

A similar approach was tested for *A.ochraceus* identification. The use of species specific primers for *A. ochraceus* (OCRA1/OCRA2), designed on the basis of ITS (internal transcribed spacers of rDNA units) sequence comparisons obtained from *Aspergillus* strains as indicated by Patino et al. (*15*), resulted in the obtaining of a single fragment of 400 bp in two out of five strains suspected as *A.ochraceus* (fig. 4).

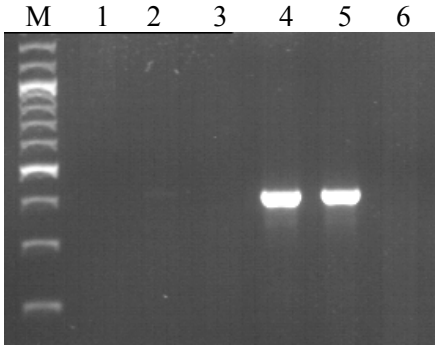

Figure 4. Agarose gel analysis of PCR products obtained with the primer pair OCRA1/OCRA2. 1, 10 = marker ADN; lanes 1 – 5 = *A.ochraceus;* lane 6 = *A.niger*

It is obvious that the primers used in experiments allow the discrimination among the species considered. For this reason, the PCR reactions utilized DNA extracted from mixtures of fungi (from plates containing non-purified cultures of fungi isolated from bakery). Acceptable quantity and quality of fungal DNA was obtained by applying the method of Richard et al (*17*). Eight samples of total fungal DNA, corresponding to isolation sites (production areas), were used in the experiments. When multiplex PCR using the primer pairs for *A.flavus, A.niger* and *A.fumigatus* was applied, specific amplicons were obtained (fig.5).

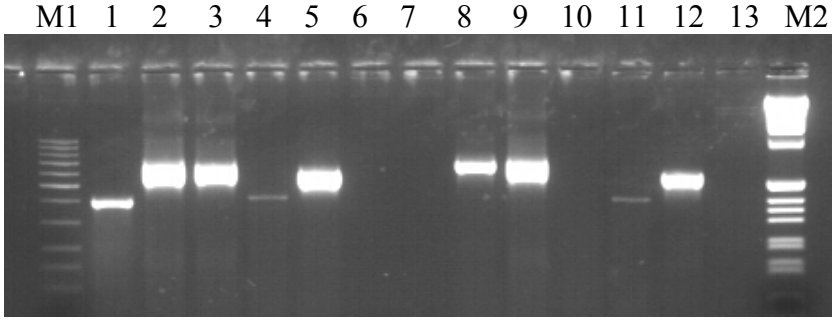

Figure 5. Species-specific single and multiplex PCR performed with total DNA isolated from mixed fungal cultures. M1 – 100bp DNA ladder (Roth); M2 – 1kb DNA ladder (Gibco BRL); lane 1 – single PCR of total DNA from pre-cooling with OCRA1/OCRA2; lane 2 – multiplex PCR of total DNA from pre-cooling area; lane 3 - multiplex PCR of total DNA from kneading room; lane 4- single PCR of total DNA from passage hall; lane 5 - multiplex PCR of total DNA from passage hall; lanes 6 – 7 – single and multiplex PCR, respectively, of total fungal DNA from cooling area; lanes 8 and 10 – multiplex and single PCR, respectively, of total fungal DNA from fermentation room; lanes 9 and 11 - multiplex and single PCR, respectively, of total fungal DNA from slicing and packing area; lane 12 – *A.niger*; lane 13 – multiplex PCR of total fungal DNA from package/storage room.

It was shown that in samples where *A.flavus* and *A.niger* were detected simultaneously, the amplicon detected after multiplex PCR is thicker (spanning the size of approximately 600 and 680 bp) than in those obtained for each separate species, suggesting the presence of both specific amplicons.

Similar results were obtained with primers for *A.ochraceus*: unique amplicons of 400 bp were identified in two of the samples tested (from passage hall and from slicing and packing area, respectively) (fig.5). No detectable amplicons were obtained both by single or multiplex PCR using total DNA from cooling room, dough partition area or package/storage room where the contamination with aspergili was reduced or absent.

These results are highly correlated with the classical identification performed at the beginning of the experiments. However, for two samples in which *A.fumigatus* was considered present, the molecular techniques did not confirm it. The same occurrence was

observed for another sample where *A.ochraceus* was identified; the specific PCR reaction was negative for these species.

Screening of aspergili for mycotoxin production

Among 18 aspergilli selected at random (5 strains of *A. flavus,* 5 strains of *A. niger,* 5 strains of *A. ochraceus* and 3 strains of *A. candidus*) from different bakery areas, only three were mycotoxin producers, as was revealed by the fluorescence surrounding the colonies grown on coconut based medium (assayed under UV light), after 48h-72h of growth. These strains belong to *A. ochraceus* and *A. niger*. The coconut medium is recommended for aflatoxin producer screening and several papers have described its usefulness for ochratoxin detection (*19*).

The absence of the fluorescence associated with aflatoxin production in *A.flavus* could be due to the accuracy of the screening method that is too low to permit the detection in these strains isolated from the bakery.

Previous reports have shown that screening for the presence of specific genes involved in aflatoxin biosynthesis pathway could be a more reliable method of aflatoxigenic aspergili identification. In order to distinguish between aflatoxin - producing strains and non - producing strains by molecular techniques it is necessary to detect the PCR products corresponding to four genes: *aflR, nor1, ver1* and *omtA* (*2, 18*). These primers were used in screening experiments applied to all 18 aspergili in order to distinguish the potential aflatoxin producing strains.

The primer sets were chosen for detection of *nor1* gene (encode one of the first enzymes within the aflatoxin biosynthesis pathway) and for *aflR* gene (involved in the regulation of aflatoxin biosynthesis). They were used in a multiplex PCR reaction. Four amplicons of 400 bp (corresponding to *nor 1* gene), 1032 bp (for *aflR* gene), respectively, were expected to be obtained (*8*).

DNA isolated from all 18 strains of aspergili was used in the molecular screening experiments. Among aspergili tested, amplification products were obtained only for *A. flavus* and the amplicon of 400 bp corresponding to *nor1* gene was observed in all of these strains (fig.6).

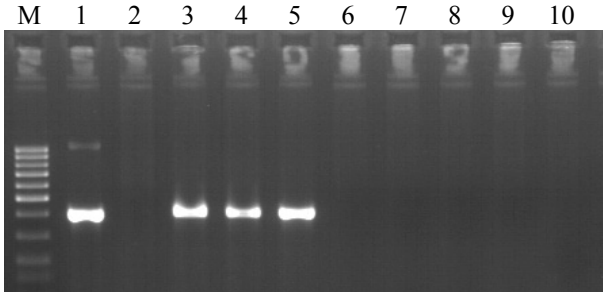

Figure 6. Agarose gel analysis of PCR products obtained with the primers specific for *nor1* and *aflR* genes, in a multiplex PCR reaction. M = ladder marker (100 bp); lanes 1 - 5 = Asp; lanes 6 - 10 = An

The amplicon of 1032 bp, corresponding to regulatory gene *aflR* was detected only in one strain previously confirmed to be *A.flavus*. This gene plays an important role in the aflatoxin biosynthesis pathway by regulating the activity of other structural genes such as *omt A, ver 1* and *nor1* (*2*). Despite the absence of fluorescence on coconut medium cultivated with *A. flavus* strains, the simultaneous presence of both PCR products corresponding to *nor1* and *aflR* genes suggests that at least one strain of *A.flavus* could produce aflatoxin.

These results suggest that confirmation of the aflatoxin producing capacity of the aspergili isolated from bakeries should be done, in the future experiments, by combining molecular techniques with the biochemical ones (detection and identification of aflatoxin by chromatography methods).

Conclusions

In conclusion, the PCR method allowed identification of three species of potential toxigenic fungi: *A flavus, A.niger* and *A.ochraceus*, both in pure cultures and in mix samples. Moreover, molecular methods applied for the detection of some genes involved in aflatoxin biosynthesis led to the identification of aflatoxigenic strains of *A.flavus*. The use of such methods could be of great interest to assess airborne fungal contamination in bakeries as well as in food products.

Acknowledgments

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References

1. AGUIRRE, L., S. F. HURST, J.S. CHOI, J.H. SHIN, H.P. HINRIKSON, C.J. MORRISON, Rapid Differentiation of *Aspergillus* Species from Other Medically Important Opportunistic Molds and Yeasts by PCR - Enzyme Immunoassay, J. Clinical Microbiol., vol. 42, pp. 3495 – 3504, (2004).
2. CRISEO, C., BAGNARA, A, BISIGNANO, G., Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group, Lett. Appl. Microbiol., vol. 33, pp. 291 – 295, (2001).
3. DAVIS, N.D., IYER, K., DIENER, U.I., Improved method of screening for aflatoxin with a coconut agar medium, Appl. Environ. Microbiol., vol. 53, pp. 1593 – 1595, (1987).
4. FRISVAD, J.C., LARSEN, T.O., DE VRIES, R., MEIJER, M., HOUBRAKEN, J., CABANES, F.J., EHRLICH, K., SAMSON, R.A., Studies in Mycology, vol. 59, pp. 31 – 37, (2007).
5. HALSTENSEN, A.S., Species-specific fungal DNA in airborne dust as surrogate for occupational mycotoxin exposure, Int. J. Mol. Sci., vol. 9, pp. 2543 – 2558, (2008).
6. HINRIKSON, H.P., HURST, S.F., AGUIRRE, A., MORRISON, C.J., Molecular methods for the identification of *Aspergillus* species, Medical Mycol.Suppl., vol. 43, pp. 5129-5137, (2005).
7. KANBE, T., YAMAKI, K., KIKUCHI, A., Identification of the pathogenic *Aspergillus* species by nested PCR using a mixture of specific primers to DNA topoisomerase II gene, Microbiol. Immun., vol. 46, pp. 841 – 848, (2002).
8. KONIETZNY, U., GREINER, R., The application of PCR in the deection of mycotoxigenic fungi in foods, Brazilian J. Microbiol., vol. 34, pp. 283 – 300, (2003).
9. KUMAR. M., P. K. SHUKLA, Use of PCR Targeting of Internal Transcribed Spacer Regions and Single-Stranded Conformation Polymorphism Analysis of Sequence Variation in different Regions of rRNA Genes in Fungi for Rapid Diagnosis of Mycotic Keratitis, J. Clin. Microbiol., vol. 43(2), pp. 662 – 668, (2005).
10. LOGOTHETI,M., KOTSOVILI-TSELENI,A., ARSENIS,G., LEGAKIS,N.J., Multiplex PCR for the discrimination of *A.fumigatus, A.flavus, A.niger* and *A.terreus*, J.Microbiol.Methods, 76, 209-211, (2009).
11. MANESCU SERGIU, Microbiologie sanitara, Edit. Medicala, Bucuresti, (1989), pp.31 – 32
12. MIRHENDI, H., DIBA, K., KORDBACHEN, P., JALALIZAND, N., MAKIMURA, K., Identification of pathogenic *Aspergillus* species by PCR restriction enzyme method, J.Med. Microbiol., vol. 56, pp. 1568 – 1570, (2007).
13. NIESSEN, L., PCR-based diagnosis and quantification of mycotoxin-producing fungi, Internat. J. Food Microbiol., vol. 119, pp. 38 – 46, (2007).
14. PALMAS, F., COSENTINO, S., CARDIA, P., Fungal air-borne spores as health risk factors among workers in alimentary industries, European J. Epidemiol., vol. 5, pp. 239 – 243, (1989).
15. PATINO, B., SALGADO, A.G., GONZALES-JAEN, M.T., VASQUEZ, C., PCR detection assays for the ochratoxin-producing *Aspergillus carbonatius* and *A. ochraceus* species, Internat. J. Food Microbiol., vol. 104, pp. 207 – 214, (2005).
16. RAHIMI,P., SHARIFNABI,B., BAHAR,M., Detection of aflatoxin in *Aspergillus* species isolated from pistachio in Iran, J.Phytopathol., 156, p.15-20, (2008).
17. RICHARD,E., HEUTTE,N., BOUCHART,V., GARON,D, Evaluation of fungal contamination and mycotoxin production in maize silage, Animal Feed Sci.Technol., 148, pp.309-320, (2009).
18. SCHERN, B., PALOMBA, M., SERRA, D., MARCELLO, A., MIGHELI, Q., Detection of transcripts of the aflatoxin genes *aflD, aflO* and *aflP* by reverse transcription -polymerase chain reaction allows differentiation of aflatoxin - producing and non - producing isolates of *A.flavus* and *A. parasiticus*, Internat. J. Food Microbiol., vol. 98, pp. 201–210, (2005).
19. TORELLI, E., FIRRAO, G., LOCCI, R., GOBBI, E., OchratoxinA - producing strains of *Penicillium spp.* isolated from grapes used for the production of passito wines, Internat. J. Food Microbiol.,vol. 106, pp. 307 – 312, (2006).
20. WHITE T. J., T. BRUNS, S. LEE, J. TAYLOR, PCR Protocols: A Guide To Methods And Applications, ed. M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Academic Press, San Diego, 1990, pp. 315–322.