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# Molecular identification of toxigenic *Aspergillus* and *Fusarium* species isolated from traditional cheese wagashi produced in Benin

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## Abstract

Traditional cheese *wagashi* is an important source of animal proteins of excellent nutritional values mainly for low income populations in Benin. It's often used in replacement of meat and fish in different dishes. Despite its importance, *wagashi* can be contaminated by various toxigenic mould strains capable of producing mycotoxins with advert effects on consumers' health. It is therefore, necessary to provide a clear picture of the potentially toxigenic mould strains that colonizes *wagashi*. The study aimed to identify toxigenic *Aspergillus* and *Fusarium* species isolated from traditional cheese wagashi produced in Benin in order to assess the associated health risks to consumers. Preliminary isolation and identification of seven toxigenic mould isolates were carried out using conventional microbiological, macroscopic and stereomicroscopic methods. The isolates were further confirmed by PCR using species specific primers. The study revealed that four of the seven isolates namely *Aspergillus flavus, A. ochraceus, Fusarium verticallioides, F. poae* previously identified by conventional methods were confirmed by PCR. These results showed the limits of conventional methods in microbial identification and revealed that *wagashi* produced in Benin contains toxigenic strains of moulds that can affect its safety with risks of hazardous mycotoxins ingestion by consumers.

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## Introduction

Fungal contamination of food products is a chronic problem in developing countries with negative influence on their safety, quality, quantity and nutriment contents as well as their monetary value. Studies revealed that pathogenic fungi can provoke a reduction of about 20% of food commodities (Tian *et al.*, 2011; Prakash *et al.,* 2012). Apart from their role in the deterioration of food, toxigenic fungi strains represent a serious risk to consumers because of the production of dangerous secondary metabolites, like mycotoxins (Zohra and Fawzia, 2013). The growth of moulds on foods can lead to their discoloration, their poor presentation and the *in situ* production of volatile compounds of unpleasant odour (Filtenborg *et al.*, 1996; Nasser, 2001). Moreover, moulds can produce some allergenic compounds and mycotoxins that can contaminate food and thus affect consumer's health (Ngueface *et al.*, 2004). It was reported that mycotoxins can induce serious threats to human health through hepatic, renal, nervous and carcinogenic affections (WHO/IARC, 2002).

In Benin, traditional cheese locally called wagashi is an important source of animal protein in human nutrition and can contribute to the reduction of protein deficiency among its consumers (Kèkè *et al.*, 2008). However, wagashi is produced and kept in poor hygienic conditions that can lead to its contamination by pathogenic and toxigenic microorganisms including moulds (Sessou *et al.*, 2013). The contamination of wagashi by moulds induces a loss of its quality and safety. The objective of the present study was to identify potentially toxigenic mould species isolated from wagashi produced in Benin so as to evaluate the associated risks to consumers.

## Material and methods

*Identification of mould isolates using conventional methods*

Mould colonies were isolated from traditional cheese wagashi following ISO 21527-1: 2008 standards on Dichloran Rose Bengal Chloramphenicol agar [Rose-Bengal Chloramphenicol Agar Base (CM0549 Oxoid, LTD Basingstoke, Hampshire, England), Chloramphenicol supplement (SR0078E, Oxoid), Dichloran (FLUKA Analytical Lot # SZB8239XV Sigma-ALORICH Product of Germany)]. The isolates were purified and identified. The purification of the isolates was performed by touches on identification media: Malt Extract Agar and Czapeck Yeast autolysate Agar. Their identification by conventional method was based on their cultural, macroscopic and stereomicroscopic characteristics using the keys of Samson *et al.* (1995) and Pitt and Hocking (2009).

*Identification of toxigenic mould isolates by PCR*

*Genomic DNA extraction*

DNA was extracted from fungal isolates based on methods described by Erland *et al.* (1994) with minor modifications. Briefly, 50 mg of fungal mycelium was scraped from 10-days mould isolates cultured on PDA media, frozen at -70°C for 48 hours and thawed at ambient temperature. This was ground with 200 mg of sterile sea sand and 800 µL of Tris EB buffer (200 µM TrisHCl, pH 8.5; 25 mM EDTA pH 8.0; 250 mMNaCl; 2% SDS) preheated at 60°C. The mixture was incubated at 65°C for 90 minutes. 700 µl of phenol Tris saturated was added to the suspension incubated at 65°C and homogenised for 10 minutes. The obtained mixture was centrifuged at 14 000 rpm for 10 minutes and the supernatant was transferred into a new Ependorff tube. 700 µl of chloroform-isoamyl alcool (24:1) were added to the supernatant and the mixture was homogenized for 10 minutes and then centrifuged at 14000 rpm for 10 minutes. The obtained DNA was precipitated by addition of an equal volume of cold isopropanol to the supernatant at -20°C for 1 hour. The precipitated mixture was again centrifuged at 14000 rpm for 10 minutes and the obtained pellet DNA was rinsed twice with 70° ethanol. The pellet washed and dried for 24 hours was suspended in 50 µl of TE (10mM Tris pH 8.0; 1mM EDTA pH 8.0) and kept at -20°C for future use.

*Amplification of the extracted DNA by PCR*

PCR was performed with species specific primers (Sigma-Aldrich products, USA) of the concerned mould isolates (Table 1). The mix was prepared in

Ependorff tubes containing 25 µl of the final reaction mixture composed of: 1.25 mM $MgCl_2$, 50 mMKCl, 10 mMTris-HCl (pH 8.3), 0.2 mM of each of the four dNTP, 0.4 µM of each primer, 0.5 unit of DMSO, 0.5 unit of Taq DNA polymerase and 15 to 30 ng of template DNA. The amplification was carried out in a thermo cycler (PTC-100TM Programme Thermal Controller, MJ Research, Inc). A first multiplex PCR was conducted following conditions described by Karthikeyan *et al.* (2011) to identify species like *Aspergillus flavus, Fusaiumpoae* and *Fusarium verticillioides*. A second multiplex PCR was performed as per conditions reported by Logotheti *et al.* (2009) for the identification of the remaining species.

*Gel electrophoresis*

PCR products were revealed by electrophoresis in 2.0% (P/V) agarose with 0.2 µg/ml of ethidium bromide (Tigst *et al.*, 2005) and read under transilluminator Bio block Scientific ILLKIRCH CEDEX coupled with a gel Cam Polaroid. A molecular weight marker of 100 bp was used to appreciate the size of the amplicons.

**Results and discussion**

Seven species of mould from two different genera *Aspergillus* (05) and *Fusarium* (02) were isolated from traditional cheese wagashi produced in Benin and subjected to detection by PCR using their specific primer coding for mycotoxin production. Four of the isolated species (*A. flavus, A. ochraceus, Fusarium poae* and *Fusarium verticillioides*) were confirmed by PCR.

**Table 1.** Toxigenic mould species and specific primer sequences (Sigma-Aldrich products, USA).

| Species | ID | Sequence (5'-3') | References |
|---|---|---|---|
| *Aspergillus flavus* | PEPO1 | CGACGTCTACAAGCCTTCTGGAAA | Logotheti *et al.* (2009) |
| | PEPO2 | CAGCAGACCGTCATTGTTCTTGTC | |
| *Aspergillus niger* | PEPI1 | CCAGTACGTGGTCTTCAACTC | |
| | PEPI2 | CATCACCATGACCATCGTTTGCT | |
| *Aspergillus terreus* | ATE1 | CTATTGTACCTTGTTGCTTCGGCG | |
| | ATE2 | AGTTGCAAATAAATGCGTCGGCGG | |
| *Fusarium poae* | FP82F | CAAGCAAACAGGCTCTTCACC | Tigst *et al.* (2005) |
| | FP82R | TGTTCCACCTCAGTGACAGGTT | |
| *Aspergillus carbonarius* | $OPX7F_{809}$ | Agg CTA ATgTTgA TAA CggATg AT | Sartori *et al.* (2006) ; Fungaro *et al.* (2004) |
| | $OPX7R_{809}$ | gCTgTCAgT ATT ggA CCT Tag Ag | |
| *Aspergillus ochraceus* | OCA-V | ATA CCA CCgggT CTA ATg CA | Sartori *et al.* (2006) |
| | OCA-R | TgCCgACAg ACC gAgTgg ATT | |
| *Fusarium verticillioides* | VERTF-1 | GCG GGA ATT CAA AAG TGG CC | Karthikeyan *et al.* (2011) |
| | VERTF-2 | GAG GGC GCG AAA CGG ATC GG | |

The features of each of the PCR products are shown in Table 2. Regarding *Aspergillus niger* and *Aspergillus carbornarius*, no amplification was obtained. These results shows that they would be other strains that deserve to be investigated by sequencing. The product size obtained for *Aspergillus terreus* (between 200 and 300bp) study was different from the one reported (450 bp) by Logotheti *et al.* (2009). The amplification obtained for this last isolate denotes that it would be a strain having similarities with *Aspergillus terreus*. As the two previous one, it is important to continue investigations to elucidate this fact by sequencing to identify this strain. The presence of toxigenic strains capable of producing mycotoxins in wagashi such as *Aspergillus flavus, A. ochraceus, Fusarium poae* and *Fusarium verticillioides*, constitutes a serious threat to the health of consumers. In fact, *Aspergillus flavus* is the main source of aflatoxin production in food. Aflatoxins are responsible of acute and chronic dysfunctions in human body including hepatic and

renal damages, liver cirrhosis, cancers and teratogenic effects (Pitt and Hocking, 2009). The isolation of this specie from wagashi is attributable to the contamination of the milk that served to make this wagashi. Hayaloglu and Kirbag (2007) reported that cheeses are often colonized by *Aspergillus flavus* that is a common milk contaminant (Garrido *et al.*,2003) and produces some aflatoxins sometimes. *Aspergillus ochraceus* is one of the main contaminants of Bhutanese cheese (Sinha and Ranjan, 1991) and also isolated in many cheeses from temperate climates (Pitt and Hocking, 1997).

**Table 2.** Characteristics of the toxigenic mould species used in PCR.

| Name of the species | Obtained size (bp) | Expected size + reference |
|---|---|---|
| *Aspergillus ochraceus* | Between 200 and 300 | 260 (Sartori *et al.*, 2006) |
| *Fusarium poae* | Between 200 and 300 | 220 (Tigst *et al.,* 2005) |
| *Aspergillus carbonarius* | * | 809 (Sartori *et al.*, 2006) |
| *Aspergillus flavus* | 200 | 200 (Logotheti *et al.*, 2009) |
| *Aspergillus niger* | * | 150 (Logotheti *et al.*, 2009) |
| *Aspergillus terreus* | Between 200 and 300 | 450 (Logotheti *et al.*, 2009) |
| *Fusarium verticillioides* | 400 | 400 (Karthikeyan *et al.,* 2011) |

* : species that could not be confirmed by PCR ; - : non-obtained value.

It is a mold specie known to produce ochratoxin A, one of the most abundant food-contaminating mycotoxins (Dobson, 2017). Ochratoxin A is undesirable because it is classified by International Agency for Research of Cancer) into "Group B" as a molecule with possible carcinogenic activity in humans (Iacumin *et al.*, 2017). It was reported that some strains of *Fusarium poae*, widespread in humid tropical regions and temperate climates of the world (Leslie and Summerell, 2006) commonly recovered from cheeses (Northolt *et al.*, 1980) and identified as pathogenic agents of sorghum (Leslie *et al.*, 2005), can produce the T-2 toxin that is part of the most important haematotoxic and immunosuppressive mycotoxins (Thrane *et al.*, 2004; Pitt and Hocking, 2009).

The major mycotoxin produced by *Fusarium verticillioides* reported as frequent fungus contaminating mostly cereals and cheese sometimes (Paterson et Lima, 2015), is fumonisine B1 which is the cause of liver cancer in rats and oesophagus cancer in human (Shephard *et al.,* 2007). Overall, wagashi produced in Benin contain various toxigenic mould strains capable of producing mycotoxins with advert effects on consumers' health.

## Conclusion

The present study identified four potentially toxigenic mould species from wagashi produced in Benin. The presence of these microorganisms in this food can lead to the loss of its commercial qualities and safety due to the production of mycotoxins. It is therefore, necessary to produce and keep wagashi in very good hygienic conditions in order to avoid its contamination by these fungi so as to minimize the associated health risks to consumers.

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