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Toxigenic fungi and contamination by AFB_1 in Algerian traditional foods markets

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ABSTRACT: This work focused on the realization of a mycological and mycotoxicological study of certain foods manufactured in a traditional way (couscous and spice of *Capsicum annuum* known locally under the name of sweet hror) and marketed in the city of Bechar. The physico-chemical analyzes revealed that all the samples were poorly hydrated where the average values of relative humidity ranged between 7.23% and 13.58%. For the pH, the values varied between 5.22 and 6.95. The enumeration of the fungal flora indicated that the couscous samples (coarse and fine) represented a contamination rate of $2.92*10^2$ and $1.71*10^2$ CFU/g respectively. While, the sweet hror samples represented a higher contamination rate ($4.68*10^2$ CFU/g), with a clear dominance of the genera of *Aspergillus* (46.42%) and *Penicillium* (26.28%). Otherwise, the mycotoxicological analysis showed us that 78.55% of the *Aspergillus* isolates of the group (*flavus-parasiticus*) tested were producers of aflatoxins (B_1 and G_1) and that 86.66% of the isolates of *A. ochraceus* and 40% of *Penicillium* species, were ochratoxin A producers. In addition, the detection of mycotoxins at the sample level revealed that 63.63% of couscous samples were contaminated with mycotoxins. While sweet hror was the most contaminated (78.57%). Furthermore, the quantification of AFB_1 by HPLC-FLD for 4 samples of sweet hror revealed only one contaminated sample (21.75 µg/kg). Generally, it can be admitted that the rate of contamination by AFB_1 was too high, which can be considered a real risk to human health.

Keywords: Aflatoxins; Mycotoxins; Ochratoxin A; Couscous; Sweet hror.

1. INTRODUCTION

The artisanal production of food has always been a tradition among the people of North Africa. Among these foods are couscous and the spice of sweet red pepper (*Capsicum annuum*) known locally as sweet hror. Couscous is one of the oldest dishes developed by the inhabitants of North Africa, and is among the main dishes among Algerian families. This dish remains the most appreciated by the Algerian population despite the great food diversification [1]. In addition, sweet hror is a spice derived from processing the ripe fruits of the sweet red pepper, which is widely used in cooking as a food coloring [2]. Unfortunately, certain bad conditions during the artisanal manufacturing process of these two food products put them at risk of several fungal contaminations,

which subsequently promote the production of certain highly toxic secondary metabolites called mycotoxins, representing a real danger to public health [3].

The adverse effects of mycotoxins on human health can be both acute and chronic, causing serious diseases such as liver cancer, impaired protein metabolism, gangrene, seizures and respiratory problems [4]. Among others, the economic impact of mycotoxins includes increased healthcare costs and premature deaths [5]. According to the Food and Agriculture Organization of the United Nations, crops contaminated by mycotoxins represent a quarter of the total production of the Globe [6]. Among the factors influencing the presence of mycotoxins in food are storage conditions, which can be controlled at a little cost [7]. At a time when the production of agricultural commodities is not sufficient to meet the needs of the entire world population, the choice of destroying contaminated food is not easy. On the other hand, the cleaning of food contaminated by mycotoxins is very expensive and rarely implemented [8].

Aflatoxins and ochratoxins are the most economically important mycotoxins, although dozens of others may be associated with public health risks. Despite international attempts to implement legislation to control the presence of mycotoxins in food, its implementation has been ineffective [9].

The objective of this study is to analyze the hygienic and sanitary quality of certain traditional foods (coarse couscous, fine couscous and sweet hror) marketed in the city of Bechar by determining the rate of contamination and identifying toxigenic molds dominating the different samples, as well as the detection and quantification of mycotoxins (aflatoxins B_1 and G_1 and ochratoxin A) contaminating the different samples by two methods, namely thin layer chromatography (TLC) and high-performance liquid chromatography coupled with a detector fluorescence (HPLC-FLD), with the aim of raising awareness about the danger of mycotoxins on agriculture, the economy and public health.

2. MATERIALS AND METHODS

2.1. Sampling of traditional foods

In this study, 75 samples (25 coarse couscous, 25 fine couscous and 25 sweet hror) were randomly taken from the meadows of several herbalists in the city of Bechar (31°37′N 2°13′W) located in southwestern Algeria. The collection of samples was carried out for a whole year in order to obtain a heterogeneity of the samples. All samples (250 grams for each) were stored in sterile polythene bags at 4 °C until analyzed.

2.2. Physico-chemical analyzes of traditional foods

The physicochemical parameters that were evaluated are relative humidity (RH %) and pH, which represent the main factors that affect fungal growth.

2.2.1. Determination of relative humidity

The determination of the relative humidity was carried out following a triple process of "weighing-drying-weighing" described by Multon [10]. The samples were weighed before and after drying by heating at a temperature of 105±2 °C until they reached a constant weight (P1). The mass loss was interpreted as released moisture. To obtain good results, the weightings were carried out with great precision, and at a stable oven temperature. Relative humidity was calculated by the following formula:

$$\mathbf{RH}\ \% = \frac{(\mathbf{P0} - \mathbf{Pt}) - (\mathbf{P1} - \mathbf{Pt})}{(\mathbf{P0} - \mathbf{Pt})} \times \mathbf{100}$$

Where RH: relative humidity (%); Pt: tare weight (g); P0: tare weight with sample (g); P1: constant weight after multiple drying (g).

2.2.2. pH measurement

The degree of acidity of food products is a central topic in the food sector. To measure the pH, 5 g of each sample was added to 45 ml of distilled water, with continuous stirring, then left to stand for one hour, so that the pH measurement was carried out using a pH meter [10].

2.3. Enumeration, isolation and identification of molds

2.3.1. Total fungal enumeration

The indirect method was used to count the internal flora [11]. It consists in adding 10 g of each sample to 90 ml of sterile physiological water, then adding 2 to 3 drops of Tween 80, and the total was homogenized by stirring for 15 min. Then, from the stock solution, decimal dilutions (10^{-1} and 10^{-2}) were prepared. Three Petri dishes were streaked on the surface with 1 ml of each dilution, then incubated at 25 °C for 5-7 days. Four media were used for counting: PDAac (Potato Dextrose Agar acidified), PDArb (Potato Dextrose Agar with red bengal), MEA (Malt Extract Agar) and CDA (Czapek Dextrose Agar). The PDAac medium was acidified to a pH between 4 and 5 by adding 1 ml of 25% lactic acid. In addition, the PDArb medium was prepared according to Larpent [12] by adding a few drops of 2% rose bengal to the PDA medium in order to inhibit bacterial growth and limit the size of the fungi in the Petri dish, thus facilitating counting.

2.3.2. Purification and conservation of fungal isolates

Purification consists of aseptically transferring the different isolated fungal strains to Petri dishes containing PDAac medium. Pure strains were stored in tubes containing PDAac at 4 °C for later identification.

2.3.3. Identification of molds

As described by Haris [13], micro-culture is a technique that is based on the inoculation of mold spores on slides carried out with small squares of PDAac and covering them with coverslips. The spores were inoculated on the peripheral limits of the medium, and the whole was conditioned in a sterile and humid chamber, then incubated at 25 °C for 3 to 5 days. After incubation, the slides to which the mycelium adhered were transferred to other sterile slides containing a few drops of methylene blue, so that microscopic observations were made at magnifications ×10, ×40 and ×100 [13]. The genera were determined by cultural and microscopic characters by referring to the manual of Barnett and Hunter [14].

The identification of species belonging to the genera of *Aspergillus* and *Penicillium* was carried out by the “Single Spore” method [15,16]. It is based on the relationship between the water activity of the culture medium and the incubation temperature. For this, we inoculate a hemolysis tube filled with 2/3 of its volume of 0.2% agar to which 2 drops of Tween 80 have been added. After stirring the tube, drops of this suspension were deposited in Petri dishes spread out with different culture media. The confirmation of the species of *Aspergillus flavus* and *A. parasiticus* was carried out on a specific medium, namely *A. flavus parasiticus* Agar (AFPA). This medium allows strains to produce a yellow-orange to orange color on the reverse side of the colony. This coloration is due to the production of aspergillic acid by these two fungal species which reacts with ferrous ammonium citrate, forming a colored complex after an incubation period of 5 days at 25 °C [17].

2.4. Mycotoxicological analyzes

2.4.1. Search for strains producing mycotoxins

To assess the toxicological capital of the strains isolated from the samples analyzed, all the strains of *Aspergillus flavus-parasiticus* and *A. ochraceus*, as well as those belonging to the genus *Penicillium* were

subjected to a mycotoxin productivity test (AFB_1, AFG_1 for species of *Aspergillus flavus-parasiticus* and OTA for species *A. ochraceus* and those of *Penicillium*). Initially, the fungal strains targeted for this mycotoxicological test were cultured on PDAac medium for 5 days at 25 °C. Then, they were inoculated via 2-3 discs in flasks containing 50 ml of Yeast Extract Sucrose (YES) medium, and incubation was done at 25 °C for 14 days. After the incubation period, we eliminated the biomass formed by filtering the YES medium through Wathman filter paper, then the extraction was carried out as follows: 50 ml of the filtrate obtained for each strain was added to 100 ml of chloroform (CH_3Cl), then the mixture was subjected to magnetic stirring for 30 min. Then, the chloroform phase was separated from the aqueous phase using a separatory funnel. The experiment was repeated, successively adding 50 ml and 30 ml of chloroform to the aqueous phase recovered each time for 15 and 10 min successively, and the three chloroform phases obtained were mixed and filtered through Wathman filter paper. Note that the addition of anhydrous sodium sulphate (Na_2SO_4) makes it possible to absorb all traces of water. Finally, the chloroform phase was concentrated by evaporation using a rotary evaporator until a volume of 2 to 3 ml was obtained [18]. This filtrate was stored at 4 °C in tightly closed hemolysis tubes until chromatographic analysis.

2.4.2. Detection of mycotoxins in samples

The detection of mycotoxins in the different samples was carried out according to the protocol described by Betina [19]. For this, 50 g of each sample was added to 100 ml of the solvent (chloroform-methanol v/v), then the mixture was stirred vigorously for 30 minutes. Then, the liquid phase was separated from the pellet by filtration through Wathman filter paper previously sprinkled with anhydrous sodium sulphate to remove all traces of water. This operation was repeated by successively adding 50 and 30 ml of the solvent to the pellet recovered each time after filtration. The filtrates were collected and concentrated by evaporation using a rotary evaporator until a viscous extract of approximately 1 ml was obtained. However, in order to purify the extract obtained, it was spread on a 2% agar gel at pH 7 previously poured on Petri dishes, then solidified. The dishes were left ajar to allow the evaporation of the extraction solvent, then they were kept at 4 °C for 24 hours. After diffusion of mycotoxins inside the agar, its surface was wiped repeatedly with filter paper soaked in hexane to remove macromolecules. The agar gel was then cut into small squares and mixed with 100 ml of chloroform. The whole was stirred for 30 min and then filtered. Then, the pellet was added to 50 and 30 ml of chloroform and stirred each time for 15 and 10 min successively. The filtrate obtained was also concentrated using a rotary evaporator and stored at 4 °C in tightly closed hemolysis tubes until chromatographic analysis.

2.4.3. Chromatographic analysis (TLC)

Thin layer chromatography is a classic method, which allows the separation of compounds of a sample between two solid and mobile phases, according to their affinities for the two phases. TLC takes place on a ready-to-use silica gel chromatoplate (20 cm / 20 cm), on which two parallel lines have been drawn from the start and the finish, 1 cm from the two edges, upper and bottom of the plate. In the starting line, we deposited 20 µl of the concentrated extracts of our strains selected and suspected of synthesizing mycotoxins, as well as the extracts of our samples at intervals of 1 cm. Then, the chromatoplates were placed vertically in the migration vessel filled to a height of 0.5 cm with the migration solvent (toluene/ethyl acetate/formic acid 5/4/1 ; v/v/v), so that the constituents of the sample are eluted by the mobile phase which migrates by capillary action towards the top of the plate. Once the solvent reached the top edge limit, the chromatoplates were removed and dried in a drying cabinet. The reading of the plates was carried out in a dark room under a UV lamp at 366 nm, and the

revelation of mycotoxins (AFB_1, AFG_1 and OTA) was translated by characteristic fluorescence (blue fluorescence for AFB_1, green for AFG_1 and light blue for the OTA).

2.4.4. Incidence of AFB1 in sweet hror samples by HPLC-FLD

2.4.4.1. Extraction and purification

The samples were analyzed according to the method described by Nguyen et al. [20] AFB_1 was extracted by a mixture of acetonitrile and potassium chloride. To do this, 20 g of sample were placed in 100 ml of an acetonitrile/potassium chloride (4%) (9/1; v/v) mixture, the pH of which was adjusted to 1.5 with hydrochloric acid (HCL). The mixture was stirred for 20 min and then filtered through Whatman filter paper. The filtrate was purified by adding 100 ml of hexane, then the solution was stirred for 10 min. After the separation of the two phases using a separatory funnel, the upper phase (hexane) was discarded, while the lower phase underwent two further purifications with 50 ml of hexane. The lower phases were combined and extracted with 100 ml of a mixture of chloroform and deionized water (50/50; v/v) and mixed for 10 min. After the separation of the two phases, the lower phase (chloroform) was collected in a ground-neck flask. The upper phase was re-extracted twice with 50 ml of the mixture (chloroform-deionized water v/v). Each time, the organic phase (lower) was recovered in the flask after the separation. The recovered chloroform extracts were evaporated in a rotary evaporator at 40 °C to concentrate the AFB_1. Then, the flask was washed with 2 ml of HPLC-grade methanol to re-dissolve the AFB_1, and the suspension was filtered through a 0.45 µm filter, then dried. For chromatographic analysis, 500 µl of methanol was added to the residue.

2.4.4.2. Preparation of standard solutions

The preparation of the standard solutions was carried out according to the procedures described in the international journal of official methods of analysis [21]. Due to their toxicity, handling of the AFB_1 standards was carried out respecting all the necessary precautions (use of gloves, handling under a fume hood, etc.). In addition, to avoid mycotoxin degradation, dilute solutions are prepared immediately before HPLC analysis. For this, 1 mg of AFB_1 (Libios, France) was introduced into a 20 ml amber volumetric flask, then it was dissolved by adding methanol to the gauge line, in order to obtain a standard stock solution of AFB_1 at a concentration of 50 µg/ml. From this stock solution, a solution at a concentration 10 µg/ml was prepared, then diluted to the appropriate concentration using a methanol/water solution (20/80; v/v) for the calibration range: 0.5-3-6-8-10 µg/ml.

2.4.2.3. The conditions of HPLC-FLD

The quantification of AFB_1 was carried out according to the protocol described in the international journal of official methods of analysis [21]. For this, the components of the injected solution were separated in an Agilent 1260 type chromatograph equipped with a C_{18} silica gel column (ODS2 type; 5 µm; 25 x 0.46 mm) in reverse phase, followed by detection by fluorescence (the excitation length λex = 360 nm and the emission length λem = 450 nm). The mobile phase was made up of a mixture of water, methanol and acetonitrile (650/230/120; v/v/v) with 650 µl of concentrated acetic acid and injected at a flow rate of 0.5 ml/min. The injection volume was 20 µl, and the retention time of AFB_1 was 36 min. Finally, the AFB_1 concentrations of the samples were determined by an external calibration obtained from the AFB_1 standard.

2.5. Statistical analysis

All experiments were spotted three times. MS Excel 2007 was used to express the values as the mean ± deviation.

3. RESULTS AND DISCUSSION

3.1. Physicochemical analyzes

3.1.1. Relative humidity

Relative humidity is a primary factor for mold growth and mycotoxin production, especially in poorly hydrated foods. The average values for coarse couscous range between 12.39% and 13.58%, and for fine couscous, they vary between 11.61% and 12.71%. The analysis shows that the coarse couscous samples have a higher moisture content compared to the fine couscous samples, this is probably due to the difference in grain size, where the coarse couscous can absorb more water. It was also found that two samples of coarse couscous present average values of the humidity rate above the threshold limited by the *Codex Alimentarius* [22] for couscous which is 13.5%. This is due to a short drying time or poor conservation (non-compliant).

However, the relative humidity measurement results concerning the sweet hror samples show that all the samples have a lower relative humidity rate compared to those of the couscous (coarse and fine), whose average values vary between 7.23% and 8.78%. By comparing the relative humidity measurement results of the couscous samples (coarse and fine) with those of the sweet hror samples, we found that the couscous samples are more humid compared to the sweet hror samples. This is explained by the fact that couscous is hydrated by water during its preparation. In addition, the drying time for couscous is very short compared to that of sweet hror.

3.1.2. pH values

pH can have a critical effect on fungal growth and mycotoxin production [23]. The pH measurement results of the couscous samples (coarse and fine) analyzed indicated that all the samples had a moderately acidic pH with average values between 5.33 and 6.17 for the coarse couscous and 5.22 to 6.15 for fine couscous. Concerning the sweet hror samples, the results showed that all the samples analyzed were slightly acidic with average values between 6.54 and 6.95. The majority of molds grow at a pH between 4 and 8, with optimum growth between 5 and 6, but some molds tolerate much more acidic pHs. Therefore, many foods are much more exposed to fungal spoilage due to their acidity [24].

3.2. Mycological analyzes

3.2.1. Coarse couscous

The total fungal load, as well as the different fungal strains that appeared by the suspension-dilution method in the coarse couscous samples, testify to a high rate of contamination by a high fungal flora. This rate is around $2.92*10^2$ CFU/g. The frequency of mold isolation showed the presence of eight genera, the most dominant of which were *Aspergillus* ($1.27*10^2$ CFU/g), *Penicillium* ($0.79*10^2$ CFU/g), *Fusarium* ($0.24*10^2$ CFU/g) and *Alternaria* ($0.20*10^2$ CFU/g) which correspond to the following relative densities: 48.11%, 29.24%, 7.54% and 5.66%, respectively. The main *Aspergillus* species that appeared were *A. flavus* ($0.40*10^2$ UFC/g), *A. parasiticus* ($0.33*10^2$ UFC/g), *A. niger* ($0.23*10^2$ UFC/g), *A. ochraceus* ($0.12*10^2$ CFU/g) and *A. carbonarius* ($0.11*10^2$ CFU/g). Concerning the *penicillium* genus, the dominance was for *P. chrysogenum* ($0.22*10^2$ UFC/g), *P. aurantiogriseum* ($0.18*10^2$ UFC/g) and *P. mali* ($0.15*10^2$ UFC/g). In addition, other less dominant genera with evidence of poor storage were also present, namely *Trichoderma* ($0.16*10^2$ UFC/g), *Mucor* ($0.12*10^2$ UFC/g), *Rhizopus* ($0.10*10^2$ CFU/g) and *Cladosporium* ($0.018*10^2$ CFU/g) which correspond to the following densities: 3.77%, 2.83%, 1.88% and 0.94%, respectively.

3.2.2. Fine couscous

The results revealed a relatively low contamination rate for fine couscous samples ($1.71*10^2$ CFU/g) compared to those of coarse couscous. The frequency of mold isolation essentially indicated the presence of seven fungal genera. In addition, it was found that 71% of the samples were contaminated with the genus *Aspergillus* and 59% with the genus *Penicillium*. The dominance of the genus *Aspergillus* is clearly clear, where the contamination rate was $0.71*10^2$ CFU/g, with a relative density of 46.05%. This genus was represented by six species (*A. flavus*, *A. parasiticus*, *A. niger*, *A. ochraceus*, *A. carbonarius* and *A. fumigatus*). The relative density of the genus *Penicillium* was 25% with the presence of four species (*P. chrysogenum*, *P. aurantiogriseum*, *P. mali* and *P. crustosum*). The other genera revealed in the fine couscous samples were essentially five, namely the most dominant among them were *Fusarium* and *Alternaria* with a contamination rate of $0.21*10^2$ CFU/g (relative density = 9.21%) and $0.\ 15*10^2$ CFU/g (relative density =7.89%), respectively.

3.2.3. Sweet hror

The reading of the results of the rate of contamination by the various fungal strains isolated from the samples of sweet hror, indicated to us that the total mycoflora had an average value of $4.68*10^2$ CFU/g. However, the study of the frequency of mold isolation indicated the presence of nine genera represented by *Aspergillus* (89%), *Penicillium* (76%), *Fusarium* (41%) and *Alternaria* (35%). In addition, the dominance was for the genus *Aspergillus* ($1.47*10^2$ CFU/g) with a relative density of 45.12%, taking eight species (*A. flavus*, *A. parasiticus*, *A. niger*, *A. ochraceus* , *A. carbonarius*, *A. fumigatus*, *A. clavatus* and *A. hortai*), followed by the genus *Penicillium* ($1.15*10^2$ CFU/g) with a relative density of 26.21%, with six species (*P. chrysogenum*, *P. aurantiogriseum*, *P. mali*, *P. crustosum*, *P. citrinum* and *P. rubens*). Furthermore, the presence of other genera namely *Trichoderma* (20%), *Mucor* (16%), *Rhizopus* (14%), *Cladosporium* (1%) and *Paecilomyces* (0.5%) was recorded in the samples of sweet hror.

In general, the expression of 9 different genera of moulds, representatives of both the field and storage flora, showed us the appearance of a particularly high percentage for species belonging to the genera of *Aspergillus*, *Penicillium* and *Fusarium*. According to Cahagnier et al. [25], the most frequent mold species found in food belong to the genera *Aspergillus*, *Penicillium* and *Fusarium*. They are considered field and storage contaminants.

Contamination of couscous can be caused by spores disseminated by the air if the sale of this food is anarchic, as well as the storage conditions are unfavorable. Generally, the storage conditions of the raw ingredients are poor, and they are often deposited directly on the ground without protection against agents of deterioration. In addition, the origin of the contamination of sweet hror samples by these fungal genera comes from the field, where the fresh sweet pepper fruit is contaminated by saprophytic fungi such as mucorales which settle at the level of burns or drilled holes by insects. Furthermore, insects represent the main vectors of mold spores in the field and in storage areas, through their degradation of the wall of fresh sweet pepper fruits, which will subsequently favor contamination by molds and the production of mycotoxins. Also, the mites represent the important vectors of the spores, they live on the moldy food, recover and then transport the spores on the surface of their body and in their digestive tract [26].

In addition, the culture media used gave us variable growth, this may be explained by the difference in their composition and the choice of substrates preferred by the fungal strains, as reported by the European and Mediterranean Organization for the Protection plants, that the PDA medium is prepared on the basis of organic element and the CDA medium is prepared on the basis of mineral element [27]. In addition, given the great

richness in nutrients (maltose, fructose, glucose, sucrose, etc.), malt extract agar is widely used for the enumeration of molds in food products, it is also suitable for the maintenance strains [28].

3.3. Identification of molds

According to Barnett and Hunter [14], based on the study of characters macroscopic (color, colony appearance, back of boxes, etc.) and microscopic (shape of thallus and spores, etc.) of the isolated fungal strains, we have identified nine fungal genera (Figures 1, 2).

The identification of species belonging to the genera of *Aspergillus* (*A. flavus*, *A. parasiticus*, *A. ochraceus*, *A. niger*, *A. carbonarius*, *A. hortai*, *A. fumigatus* and *A. clavatus*) and *Penicillium* (*P. chrysogenum*, *P. aurantiogriseum*, *P. mali*, *P. crustosum*, *P. citrinum* and *P. rubens*) was carried out following the inoculation of these species on different culture media at different temperatures, referring to the identification keys of Pitt [15], Ramirez [16] and Pitt and Hocking [17]. The determination of the species was carried out after reading the diameters, the color of the mycelia and the metabolites produced.

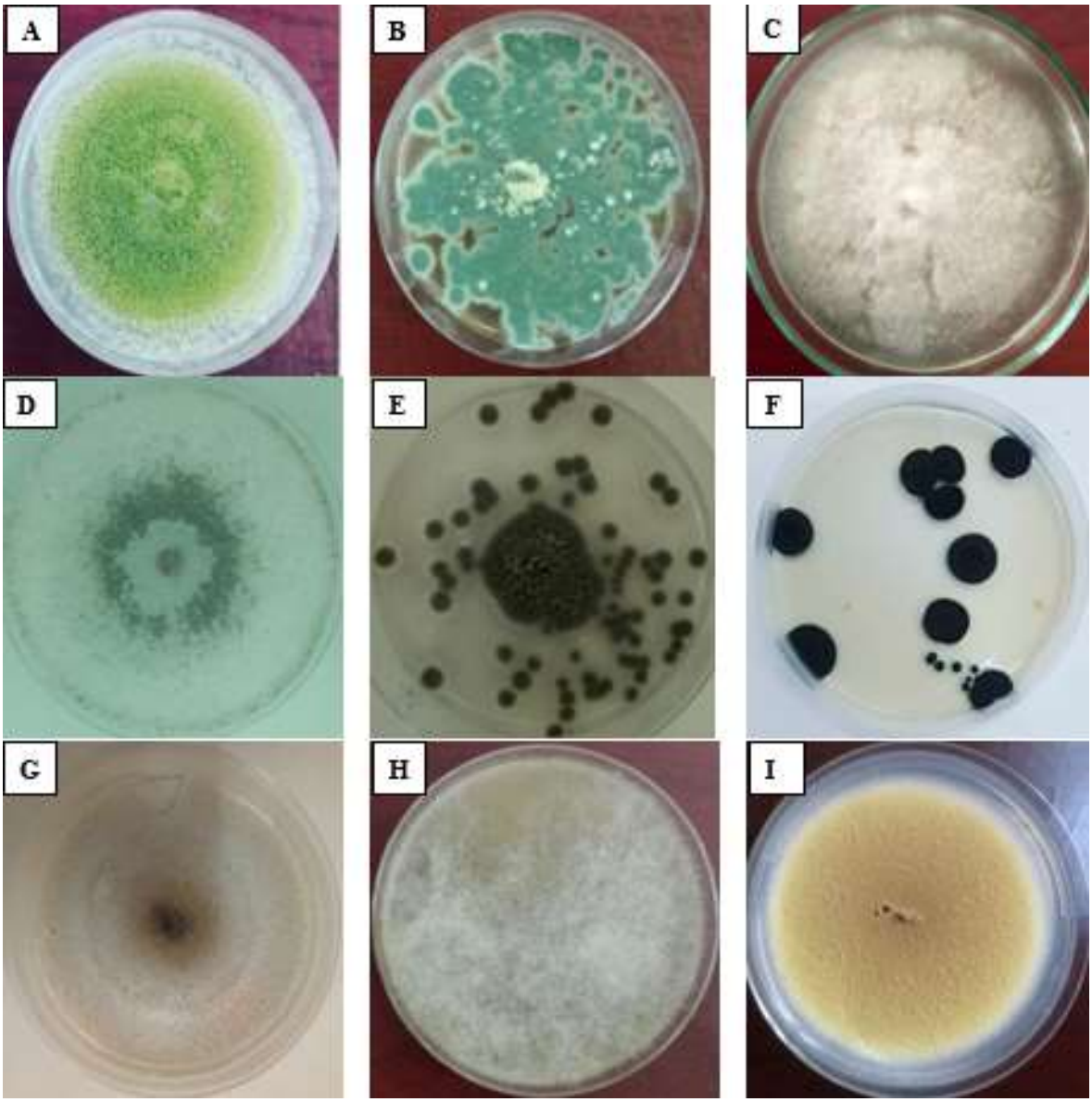

Figure 1. Macroscopic identification of nine fungal genera. A: *Aspergillus spp*, B: *Penicillium spp*, C: *Fusarium spp*, D: *Trichoderma spp*, E: *Alternaria spp*, F: *Cladosporium spp*, G: *Mucor spp*, H: *Rhizopus spp*, I: *Paecilomyces spp.*

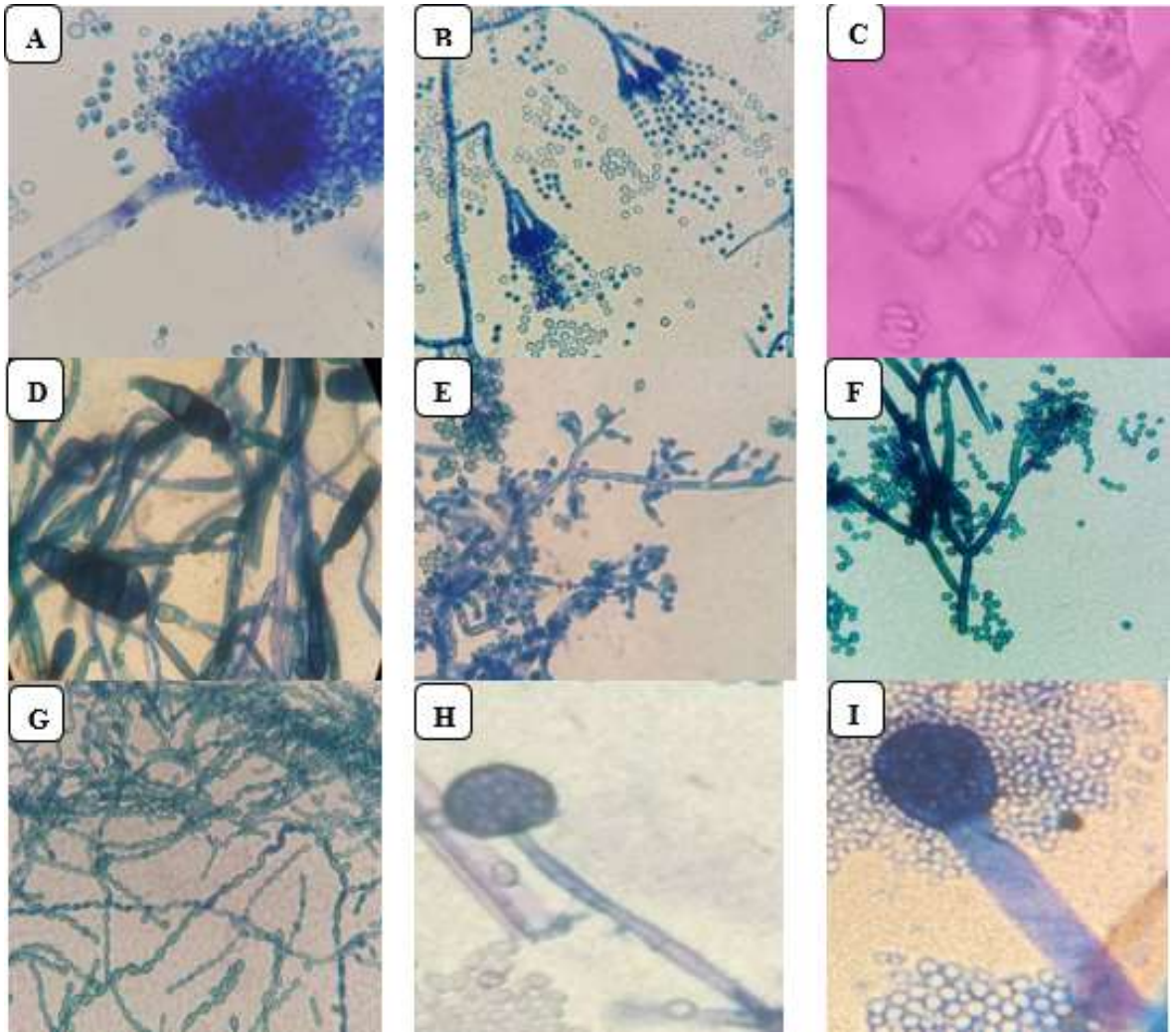

Figure 2. Microscopic identification (x 100) of nine fungal genera by the micro-culture method. A: *Aspergillus spp*, B: *Penicillium spp*, C: *Fusarium spp*, D: *Trichoderma spp*, E: *Alternaria spp*, F: *Cladosporium spp*, G: *Paecilomyces spp*, H: *Mucor spp*, I: *Rhizopus spp.*

3.4. Mycotoxicological analyzes

3.4.1. Search for strains producing mycotoxins

The study of toxigenic power carried out for 14 strains of *Aspergillus* of the group (*flavus-parasiticus*) revealed that 78.55% of the strains tested were producers of aflatoxins (B_1 and G_1). Three strains (S_1, S_2, S_6) were producers of AFB_1, and three strains (S_4, S_8, S_9) were producers of AFG_1 and five strains (S_3, S_5, S_7, S_{10}, S_{13}) were producers of both AFB_1 and AFG_1. Noting that, three strains (S_{11}, S_{12}, S_{14}) were not producing AFs (Table 1).

Table 1. Summary of aflatoxin gene group strains (*flavus-parasiticus*).

Type of strains	Types of AFs	Number of strains	Percentage	
Producers	AFB_1	3	21.42%	78.55 %
	AFG_1	3	21.42%	
	AFB_1 et AFG1	5	35.71%	
No producers	-	3	21.4%	

During recent work carried out on 11 fungal strains of the genus *Aspergillus* of the group (*flavus-parasiticus*), the results showed that six strains produced mycotoxins. This means that 54.54% of the strains were toxigenic [29]. In addition, among several studies carried out on the analysis of the toxinogenic potential of the *Aspergillus* group (*flavus-parasiticus*), Khaldi [30] reported that 60% of the tested strains produced AFs, respectively.

Furthermore, OTA is considered a renal carcinogen upon long-term exposure. It has been classified in group 2B as "possibly carcinogenic to humans" by the International Agency for Research on Cancer [31]. It is produced mainly by *Aspergillus ochraceus* and several other species of the genus *Penicillium*. Our results concerning the OTA production test carried out for 15 strains of *A. ochraceus*, revealed that 86.66% of these strains were OTA-producing. In addition, the results of the ochratoxinogenicity test of 15 other strains belonging to the genus *Penicillium* revealed that six strains (40%) produced OTA.

3.4.2. Detection of mycotoxins in samples

The qualitative test by TLC for the detection of mycotoxins in the samples was found to be positive. This is explained by the fact that the toxigenic molds isolated from these samples produced mycotoxins, in addition the optimum temperature for toxigenesis is close to the optimum temperature for fungal growth [32]. The test carried out for 11 coarse couscous samples revealed that 7 samples (63.63%) were contaminated with the targeted mycotoxins. In addition, the qualitative test revealed that among the 11 fine couscous samples tested, 7 (63.63%) were contaminated with mycotoxins. Moreover, sweet hror was the most contaminated (78.57%). The results showed that 11 samples were contaminated with mycotoxins out of 14 samples tested. The absence of mycotoxins in some samples can be explained by mycotoxin levels below the detection threshold with the TLC method [33].

3.4.3. Incidence of AFB1 in sweet hror samples by HPLC-FLD

3.4.3.1. Determination of retention time

The chromatographic conditions by HPLC-FLD were defined by the use of a standard solution of AFB_1 of known concentrations. This standardization evaluated the retention time of AFB_1 at around 37 minutes. This retention time is used to reveal the AFB_1 during its assay in the samples to be analyzed (Figure 3).

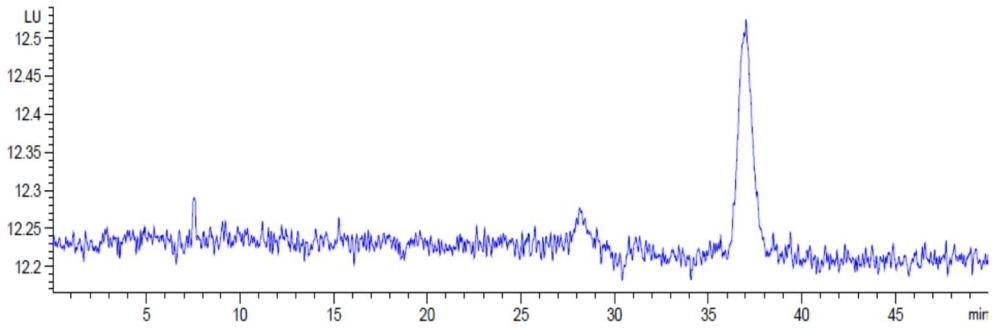

Figure 3. AFB_1 standard peak.

3.4.3.2. Determination of the calibration curve

The quantification of AFB_1 is based on the establishment of a calibration curve expressing a linear relationship between the AFB_1 standards of known concentrations (from 0.5 to 10 µg/ml) and the areas of the peaks of the signals emitted (Area = f ([AFB_1])). The calibration curve shows a good linear correlation with an

excellent correlation coefficient of 0.991, which reflects a very good proportionality between the areas of the signals emitted and the range of standards used.

3.4.3.3. Quantification of AFB_1 in sweet hror samples

AFB_1 was found in 25% (1/4) of the samples analyzed, at a rate of 21.75 µg/kg. For the other samples (3/4), the contamination was in the form of traces. It can generally be admitted that the AFB_1 contamination rate was very high if we compare the results obtained with the standard of 10 µg/kg set by Algerian legislation for spices intended for consumption. Although the other samples are contaminated with traces of AFB_1, the quantity detected in the first sample can be considered a real risk for human health. Pepper has been widely reported to be frequently contaminated with AFB_1 in different countries around the world. In Europe, in 2017 and 2018 alone, 41 cases of pepper contamination were reported, where 30 of them were classified as rejections at the border, 5 as alerts and 6 as notifications of information [34].

Furthermore, Singh and Cotty [35], assessed the AFB_1 contamination in chillies from the United States of America and Nigeria markets. These authors compared the levels of AFs in peppers from the two countries, and found that samples purchased in Nigeria were more contaminated than those purchased in the United States of America. AFB_1 was detected in 64% of peppers in the United States markets and in 93% of Nigerian peppers. Only 2% of United States samples exceeded the regulatory limit of 20 µg/kg for total AFs, while the highest concentration detected was 94.9 µg/kg. Moreover, AFB_1 concentrations were significantly higher in Nigerian pepper, of which the most contaminated sample contained 156 µg/kg of AFB_1. Additionally, approximately 38% of peppers in the United States were contaminated with > 5 µg/kg of AFB_1 (mean = 11.1 µg/kg), and based on European Union, all these peppers would be refused for import into the European Union.

However, despite the excellent sensory characteristic of Pakistani hror, it has lost its place in the international market due to mycotoxin contamination [36]. Previous studies have already reported higher levels of AFs than those established by the European Union [37]. Furthermore, prepared hror has been shown to be more susceptible to AFs contamination than fresh fruit [38].

In general, AFs can be produced at any stage of the sweet pepper production chain. In the field, fruits are more sensitive to AFs contamination during the summer [39]. Moreover, even when properly stored, it is still possible to detect traces of mycotoxins in pepper samples [40,41]. Moreover, Ozkan et al**.** [42], reported that in crushed pepper samples, AFB_1 levels varied depending on the raw material collection season.

Good cultural practices, especially after harvest, can minimize mycotoxin contamination. Sweet pepper fruits are often moistened by sprinkling them with water before marketing. This practice is likely to promote mold growth. Therefore, advice on post-harvest handling of peppers to farmers as well as traders can help minimize mold growth. Since *capsicum* is an important, high-value export product, we expect farmers to respond to any improved processing methods that can result in a safe and quality product. However, consumer awareness of the presence of mycotoxins in peppers and its derivatives can encourage producers and traders to market mycotoxin-free products [43].

4. CONCLUSION

Given the daily and/or occasional consumption of couscous and sweet hror, which is a very noble dish in Algeria, as a preventive measure, certain advice must be taken seriously to reduce the risks associated with the presence of mycotoxins in food:

- Consumer awareness on the danger of mycotoxins on agriculture, the economy and public health;
- Comply with hygiene rules (packaging, warehousing and storage conditions, etc.).

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