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Incidence of mycobiota and aflatoxin B1 in Algerian feed

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Incidence of mycobiota and aflatoxin B1 Algerian feed

Abstract

The presence of fungi and aflatoxin B1 (AFB_1) in 101 animal feed samples randomly collected from different vendors and factories in Algeria was investigated. For fungi, the main genera isolated were *Aspergillus, Penicillium* and *Fusarium.* Furthermore, the 459 strains of *Aspergillus* section *Flavi* were screened for their ability to produce aflatoxins and cyclopiazonic acid. 49% of the strains produced AFB_1. The highest incidence of aflatoxigenic strains was recorded in maize (61%) and ground poultry feed (60%). The presence of AFB_1 in feed samples was evaluated using HPLC-FLD. The obtained data showed that 36.6% of samples were contaminated in the range of 0.34 to 171.06 µg/kg. Six samples exceeded the Algerian maximum limit of 20 µg/kg for AFB_1. This study highlights the potential presence of aflatoxigenic strains belonging to section *Flavi* and AFB_1 in animal feed at post-harvest in Algeria, strategic information for the Algerian policies makers.

Keywords: Animal feed, *Aspergillus* section *Flavi*, aflatoxin B_1, Algeria

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1 Introduction

Feed products are usually low water activity nutrient mainly subjected to contamination by pathogenic mycoflora (Streit et al., 2012; Yang and Heinsohn, 2007). The presence of fungi leads to a reduction in yield, quality and organoleptic properties of feed and results in significant economic losses. Some fungi produce mycotoxins including aflatoxins, fumonisins, ochratoxins, trichothecenes and zearalenone (Dijksterhuis and Samson, 2007). This contamination can lead to cancer, immunodeficiency, weight loss and death for both humans and livestock. In addition, some mycotoxins (e.g. aflatoxins) can be bio-transformed by the animal metabolism and accumulate in meat, milk and eggs (Fink-Gremmels and van der Merwe, 2019; Santos Pereira et al., 2019).

Animal feeds are very susceptible to aflatoxins contamination (Peles et al., 2019). These mycotoxins are secondary metabolites produced by species of *Aspergillus* section *Flavi*. *Aspergillus flavus* and *A. parasiticus* can produce B1 (AFB_1), B2 (AFB_2), G1 (AFG_1) and G2 (AFG_2) and are the most common species associated with crops contamination. Although AFB_1 is considered as the most toxic and prevalent, all the 4 aflatoxins are validated human carcinogens (Group I, International Agency for Research on Cancer) (IARC, 2002).

Moreover, increased caution is needed on dairy cattle. Indeed, AFB_1 is metabolised into aflatoxin M1 (AFM_1), another group I carcinogen accumulating in cow milk. As aflatoxins may pose significant threats to the animal production industry worldwide, regulations have been established in more than 120 countries worldwide (Wu and Guclu, 2012). In Algeria, the legal limit of AFB_1 in feed materials has been set at 20 µg/kg (FAO, 2004).

As a results of the regulations, aflatoxins are impacting the world import and export markets of several products (Nidhina et al., 2017). In Algeria, the general ingredients composing animal feeds such as cereals (maize and barely), oil seeds (soybean), fiber sources (wheat bran), limestone and vitamin-mineral premix are mainly imported and can be contaminated by aflatoxins (Bessaoud, 2019). In addition, animal-based food products derived from cattle and poultry industry constitute an important portion of the current Algerian diet and are the main impacted by aflatoxin intoxification.

In Algeria, mycotoxin occurrence data are still sparse and limited to food, such as wheat, dried fruits and spices (Ait Mimoune et al., 2016, 2018; Azzoune et al., 2016; Guezlane-Tebibel et al., 2013; Riba et al., 2008, 2010, 2013; Zebiri et al., 2018). For feed, no occurrence data are yet available. The first insight for Algerian animal feed was recently

given on the occurrence of *Aspergillus* section *Flavi* thanks to Bouti et al. (2020). However, to date, there are no reports on the levels of aflatoxins in these products. Therefore, the present study was undertaken to investigate the presence of mycobiota and natural occurrence of AFB_1 in raw and finished materials meant for poultry and cattle feed consumption in Algeria.

2 Materials and methods

2.1 Sampling

A total of 101 animal feed samples from several localities in Algeria were randomly collected between 2013 and 2015. The samples were obtained from animal feed vendors and feed milling companies and included raw feed materials; maize (n=29), soybean (n=10), barley (n=5), wheat bran (n=7), as well as finished poultry and cattle feed materials; powdered (n=29) and pelleted (n=21). Each sample consisted of three subsamples collected from random parts of the bag for a final sample weight of 1 Kg. The samples were then homogenized and milled using a kitchen grinder (Moulinex, France). For mycological study, samples were analyzed upon arrival and from each sample, a 100 g subsample was randomly selected and kept at 4 °C for subsequent AFB_1 analysis.

2.2 Chemicals

The used reagents and solvents in this study were of liquid chromatography or analytical grade. In all analytical steps, Milli-Q water produced by Millipore Academic System was used. Aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) standards (Sigma-Aldrich, France). Standard solutions were prepared in methanol and stored in the dark at -20 °C. The cyclopiazonic acid (CPA) standards (Sigma Aldrich, Steinheim, Germany) was prepared in HPLC grade acetonitrile at a concentration of 2 mg/mL.

2.3 Fungal isolation

Fungal isolation and enumeration was done by dilution plating (Pitt and Hocking, 2009). Briefly, 10 g of each sample was shaken in 90 mL of sterile distilled water. Serial dilutions were then prepared up to 10^{-3} and an aliquot of 0.1 mL per dilution was inoculated in triplicate on plates containing Dichloran Rose Bengal Chloramphenicol (DRBC) medium. All

samples were incubated in the dark at 25 °C for 7 days. Then Colony Forming Units per gram of sample (CFU/g) were calculated.

For maize and barley kernels, the direct plating method was also used for the determination of mycobiota. From each homogenized sample, a 100 g subsample was surface disinfected (immersion in 0.5% sodium hypochlorite for 1 min), to eliminate external contaminants, followed by vigorous rinse with sterile distilled water. After disinfection, pods were plated in DRBC medium. Five plates (10 kernels each) were prepared per sample (total of 50 kernels per sample). After an incubation at 25 °C for 5 days, the results were expressed as a percentage of kernels/pods infected according to Pitt and Hocking's method (2009).

2.4 Fungal morphological identification

Filamentous fungi were identified to genus level according to Raper and Fennell, (1965) and Pitt and Hocking, (2009). After incubation, plates were examined and all the fungal colonies presenting different morphologies were subcultured on Potatoes Dextrose Agar (PDA) medium at 25 °C for 7 days in the dark. The different sections of *Aspergillus* were identified using both macroscopic and microscopic characteristics. Identification of the isolates belonging to *Aspergillus* section *Flavi* were further characterized based on the color of the colony, the presence of sclerotia and their size, and the conidia morphology observed on Czapek Yeast extract Agar (CYA), Malt Extract Agar (MEA) and *Aspergillus flavus* and *parasiticus* Agar (AFPA) media (Samson et al., 2014).

2.5 Screening of **Aspergillus** *section* **Flavi** *for aflatoxins production*

Strains belonging to *Aspergillus* section *Flavi* (n=459) were screened for aflatoxins production. All isolates were inoculated centrally on Coconut Agar Medium (CAM) and incubated at 28 °C for 7 days in the dark. Aflatoxins extraction was performed with methanol following the method described by Bragulat et al. (2001). The screening was carried out by spotting the extracts (20 µL) and aflatoxin standards (20 µL) on Thin Layer Chromatography (TLC) silica gel 60 F254 plates and with a mobile phase of chloroform: acetone (90:10, v/v). Plates were visualized under ultraviolet (UV) light (365 nm) to detect the fluorescence of aflatoxins. The Limit of Detection (LOD) of this technique was 0.5µg/kg for each aflatoxins.

2.6 Cyclopiazonic acid detection

For CPA production, all isolates were inoculated on CYA medium and incubated for 14 days at 25 °C in the dark (Gqaleni et al., 1997). Following the method of Bragulat et al. (2001), CPA was extracted and analyzed using TLC method. The detection of CPA was performed using Ethyl acetate/propanol/ammonium hydroxide (40:30:20) as developing solvent system (Fernández Pinto et al., 2001).

20 µL of CPA standard and test samples were dropped on TLC plates and dipped in methanol:oxalic acid (98:2, v/v) for two minutes. The visualization of blue purple color for CPA detection is done after plate's developpement after using Ehrlich's reagent. The TLC technique had an LOD of 1 µg/kg.

2.7 Aflatoxin B1 analysis and clean-up

2.7.1. Extraction and clean-up

For the extraction of AFB_1 from milled maize samples, 20 g were mixed in a blender at high speed with 100 mL methanol: water (80:20, v/v) solution for 5min. The extracts were filtered and 14 mL of the each filtrate was diluted by the addition of 86 mL of Phosphate-Buffered Saline (PBS) solution. Regarding the fatty matrices, the extraction of aflatoxins was performed by the addition of 100 mL methanol: water (80:20, v/v) solution containing 2 g of NaCl to 20 g of samples. After filtration, 50 mL of n-hexane were added and shacked at high speed for 5 min. The solution was allowed to stand and the hexane fraction was discarded. Thereafter, 1 mL of the aqueous fraction was mixed with 6 mL of PBS containing 8% of tween 20 (pH 7.4). Finally, all the extracts were filtered through a Whatman filter paper N°1 to remove residual turbidity. Subsequently, 10 mL from the maize extracts and 2.8 mL from the other extracts were loaded into immunoaffinity columns AflaCLEAN™ SMART (Germany). The columns were washed with 2 mL of distilled water with a maximum flow rate of 3 mL/min. The residual water was removed by flushing air through the columns and AFB_1 was slowly eluted by passing 0.4 mL of High Performance Liquid Chromatography (HPLC) grade methanol through columns at a rate of 1 to 2 drops/s in amber vials. The AFB_1 concentration was measured directly by HPLC.

2.7.2 HPLC analysis

AFB_1 concentration was determined after injection of 10 µL into an HPLC Dionex UltiMate 3000 modular system coupled with a Kobra Cell post-column derivatization (Dionex, FR). The Dionex UltiMate 3000 fluorescence detectors was used at λexc of 365nn and λem of 440 nm. The chromatographic separation was performed using a Phenomenex Kinetex C18 analytical column (250 x 4.6 mm 5 µm particle size), with a pre-column (10 x 4.3mm) at a flow-rate when operating in the isocratic mod of 1 mL/min. The mobile phase used was defined by the Kobra Cell manufacturer instructions.

The calibration curve of HPLC method for AFB_1 was constructed over concentrations ranging from 5 to 100 µg/kg. The obtained data were acquired with Chromeleon software. The LOD that was defined as a signal-to-noise ratio 3: 1, was 0.05 µg/kg.

2.9 Statistical analysis

For the distribution of fungal genera, statistical analysis was performed using Excel Stat Software through a one-way analysis of variance (ANOVA). The significance of Duncan's multiple range tests for the variable was employed in order to compare the means of fungal counts in all the analyzed raw and finished materials.

3 Results and discussion

3.1 Mycobiota analysis

The total fungal counts and identification of potential toxigenic fungi are important indicators of hygienic quality of animal feeds. In our study, the mycological analysis that was conducted by the two methods of isolation (direct plating and dilution method) revealed that all the feed samples were contaminated by fungi. Table 1 shows the result of the direct plating method in maize and barley. The highest contamination percentages were recorded in maize (86.77%) and followed by barley (78.17%). In the dilution method, mean colony counts were ranged between 2.5×10^2 and 4.7×10^4 CFU/g (Table 2). Significant differences in fungal contamination levels were observed (<0.05) in the studied substrates. The obtained results showed that milled maize, wheat bran and ground poultry and cattle feeds had mean fungal counts $>10^4$ CFU/g (Table 2). According to Good Manufacture Practices (GMP, 2008), feeds

with good microbiological quality should present a maximum of 10^4 CFU/g.Maize is a predominant cereal in feed mixtures. Our results revealed that maize was the most contaminated raw material with a contamination level reaching 4.7×10^4 CFU/g. Several studies have shown similar contamination levels in maize. In Argentina, the same results were reported by Astoreca et al. (2011) with fungal counts of 4×10^4 - 8×10^4 CFU/g.

Pereyra et al. (2011) also reported fungal contamination levels over 1×10^4 CFU/g in all the analyzed milled maize and finished pig feed samples. The use of barley in animal feeds in Algeria is limited to some producers. In our study, samples collected from National Board of Livestock Feed (Office National des Aliments de Bétail, ONAB) contained barley. These samples gave acceptable fungal counts of 7.7×10^3 According to Good Manufacture Practices (GMP, 2008). Our findings are close to those obtained by Tabuc et al. (2009) indicating a fungal count of 26.6×10^3 CFU/g.

For wheat bran and soybean samples, total fungal counts of 2.4×10^4 and 5.9×10^3 CFU/g were found, respectively. A previous study reported similar results showing values of 9×10^3 in wheat bran and 9×10^2 CFU/g in soybean (Pereyra et al., 2011).

Ground poultry and cattle feeds showed high fungal counts (4.5×10^4 and 3.3×10^4 CFU/g) compared to the pelleted poultry and cattle feeds (2.5×10^2 and 8×10^2 CFU/g). This might be due to the effect of the high temperatures of some steps in the granulation process which can greatly reduce the risk of contamination in pelleted feeds. These findings are in agreement with the report of Astoreca et al. (2011) and Monge et al. (2013) who found low contamination levels in pelleted poultry feed from Argentina. In addition, Parviz et al. (2014) reported a mean fungal contamination level of 6.4×10^4 CFU/g in poultry feed samples from Iran. In contrast, the results obtained in the study conducted by Gonzales Pereyra et al. (2012) investigating the mycobiota of cattle feed showed high fungal counts of more than 10^8 CFU/g, which exceeded the limit of fungal colonies established as a hygienic quality standard (GMP, 2008).

3.2 Distribution of fungal genera

Common types of potential toxigenic fungi were found with the two enumeration methods. The frequency and distribution of mycoflora in animal feed samples are summarized in Tables 1 and 2. *Aspergillus, Penicillium* and *Fusarium* were the primary fungal genera isolated.

With the dilution method, the highest contamination rates with *Fusarium* were recorded in maize (91.65%) followed by ground poultry feeds (64.21%), barley (60.87%), ground cattle feeds (60.75%) and soybean (52.94%). In the case of wheat bran, *Aspergillus* genus was the most frequent fungi with a percentage of 69.64%. For the pelleted form of cattle and poultry feeds, we reported 58.93% and 50% of *Aspergillus* infection, respectively.

In contrast, the direct plating method used for maize and barley, showed that the most detected genus was *Aspergillus* with the mean percentages of 58.77% and 73.17% respectively, followed by *Penicillium* (25.04% and 8.17%) and *Fusarium* (6.61% and 5.50%).

Fusarium was found to be a major contaminant of milled maize and barley when using the dilution method whereas the direct plating method showed lower contamination levels with this genus. This could be the result of the superficial disinfection method that reduced the level of contamination with *Fusarium* species in the grains (Tabuc et al., 2007).

Many studies have shown the predominance of *Aspergillus*, *Penicillium* and *Fusarium* genera in animal feeds. Ghiasian and Maghsood (2011) found that the predominant fungal genera isolated from cow feed in Iran were *Aspergillus* (37.4%) followed by *Penicillium* (23.7%) and *Fusarium* (17.5%). Furthermore, Gonzales Pereyra et al. (2012) examined Argentinean cattle feed and reported a predominance of *Fusarium* spp. (50%) followed by *Aspergillus* (37.5%) and *Penicillium* (35%). Also, Greco et al. (2014) revealed that *Fusarium* genus was the most common fungus (69.6%) followed by *Eurotium* (52.2%), *Penicillium* (45.65%) and *Aspergillus* (43.5%) isolated from poultry feed in Argentina.

3.3 Distribution of different sections in Aspergillus

Aspergillus genus analysed in this study belong to *Flavi, Terei, Nigri, Clavati, Candidi* and *Fumigati* sections. As indicated in Table 3, members of *Aspergillus* section *Flavi* were the most isolated species in samples with a relative density of 70.81%. *Aspergillus* section *Terei*, *Nigri*, *Clavati*, *Candidi* and *Fumigati* occurred in low frequencies except in maize (32.79% of *Aspergillus* section *Fumigati*), wheat bran (37.61% of *Aspergillus* section *Terei*) and ground poultry feeds (42.16% of *Aspergillus* section *Nigri*).

Such results were previously reported by del Palacio et al. (2016) in wheat silage for dairy cattle feeding in Uruguay. *A. niger* aggregate, *A. clavatus* and *Aspergillus* section *Flavi* were the most prevalent species. Data from Spain conducted by Accensi et al. (2004) on animal

feedstuffs, reported *Aspergillus* as a predominant genus in mixed feeds with 43% belonged to *A. flavus*.

Our results differ from those obtained by Gonzales Pereyra et al. (2012), who found high incidences of *A. fumigatus* species (51.6%). These authors demonstrated that the presence of *A. fumigatus* in samples increased the risk of ingestion of pathogenic spores and potential exposure to mycotoxins such as gliotoxins. In our study, the *Aspergillus* section *Fumigati* were outnumbered by the section *Flavi* (51.6%).

3.4 Identification of **Aspergillus** *section* **Flavi** *strains*

In this study, *Aspergillus* section *Flavi* isolates were identified using CYA, MEA and AFPA media (Samson et al., 2014). Macroscopic and microscopic characteristics of the fungal isolates were studied. All *Aspergillus* section *Flavi* isolates were identified to species level. Through morphological examination, the 459 strains were divided into; *A. flavus* (452 isolates) with yellow-green colonies and smooth to finely rough globose conidia, *A. parasiticus* (2 isolates) representing dark-green colonies and rough conidia and *A. tamarii* (5 isolates) with dark-brown colonies and rough conidia. Furthermore, *A. flavus* and *A. parasiticus* gave a bright orange color on the reverse side of AFPA medium and were positive for the production of aspergillic acid while *A. tamarii* was characterized by a brown color.

Our results corroborate the findings by Ezekiel et al. (2014) and Faparusi and Alagamba (2018) who described *A. flavus* as the most common species (91.8% and 43.01% respectively) isolated from feed samples from Nigeria while *A. parasiticus* had the lowest incidence. Perrone et al. (2014) evaluated the distribution of *Aspergillus* from maize in Nigeria and Ghana and reported that *A. flavus* was the most commonly isolated species (98.5%).

In our investigation, *A. tamarii* and *A. parasiticus* were recorded in very low frequencies. This was in accordance with the results reported by many authors in other countries. In Argentina, Astoreca et al. (2011) showed that 98.8% of the strains that were obtained from poultry feed samples belonged to *A. flavus* whereas only one strain of *A. parasiticus* was obtained. Accensi et al. (2004) analysed 147 feed samples and revealed the absence of *A. tamarii* for all samples collected. In another work conducted in Nigeria by Udom et al. (2012), a very low incidence of *A. tamarii* was observed in dairy cattle feed samples.

3.5 Aflatoxins, CPA and sclerotia production in **Aspergillus** *section* **Flavi**

Differences in the rate of aflatoxigenic isolates in the analyzed substrates were noticed. The percentage of aflatoxigenic strains in maize (61%) and in ground poultry feeds (60%) were very high. The lowest rate of aflatoxigenic isolates was found in pelleted cattle feeds (23%) (Table 4). Previously, Astoreca et al. (2011) reported that 62% of *A. flavus* isolated from poultry feeds were aflatoxin producers. Differences on the incidence of toxigenic isolates of *Aspergillus* section *Flavi* were observed in poultry feeds from Nigeria, with a rate of 44.4% in chick mash and 19.9% in grower mash (Ezekiel et al., 2014).

The percentage of aflatoxigenic fungi depends on several factors including the type of feed, environmental conditions and culture conditions. Agricultural products like maize are known to be good substrates for the growth of *A. flavus* and the production of aflatoxins. Several authors introduced in this paper have reported the occurrence of aflatoxigenic strains in corn and related products.

Taking into the account the combinations of aflatoxins and CPA production, strains belonging to *Aspergillus* section *Flavi* were classified into six chemotypes (Table 5). Strains able to produce AFB and CPA (chemotype I) were the most represented (48.58%). A number of 115 strains (25.05%) did not produce any aflatoxins or CPA (chemotype V). One hundred fourteen strains (114) of *A. flavus* (24.84%) and five strains of *A. tamarii* (1.09%) produced only CPA (chemotype IV). Whereas, two strains of *A. parasiticus* (0.44%) produced Aflatoxin B_1 and B_2 (AFB) and Aflatoxin G_1 and G_2 (AFG) but not CPA (chemotype VI). However, strains able to produce AFB, AFG and CPA (chemotype II) and AFB only (chemotype III) were not detected in the present study.

Approximately half (49.01%) of *Aspergillus* section *Flavi* isolated from all samples were aflatoxigenic (chemotypes I and VI). AFB was produced by 223 out of 459 isolates belonging to *A. flavus.* All *A. parasiticus* isolates (n=2) produced AFB and AFG. In contrast, *A. tamarii* strains did not produce any type of aflatoxins. These findings are supported by several authors, who mentioned that some *A. flavus* produce only aflatoxins or only CPA, both mycotoxins or neither of them and *A. parasiticus* isolates are able to produce both AFB and AFG but not CPA (Giorni et al., 2007; Razzaghi-Abyaneh et al., 2006; Vaamonde et al., 2003). In our work, *A. tamarii* strains did not produce any type of aflatoxins. These finding are consistent with previously reported results (Frisvad et al., 2019; Rodrigues et al., 2011).

The populations of *A. flavus* contain abundant amounts of non-aflatoxigenic strains. The loss of aflatoxin-producing ability in *A. flavus* could be a consequence of adaptation to a carbon-rich environment that makes the aflatoxin cluster less genetically stable (Horn and Dorner, 1999).

The ratio of CPA producers among *Aspergillus* section *Flavi* strains was very high (74.5%). In addition, strains belonging to chemotype I (AFB+ and CPA+) were quite frequent. These results suggest the possibility of co-occurrence of aflatoxins and CPA in the analysed commodities as previously reported by Astoreca et al. (2011) and Giorni et al. (2007). Other surveys conducted on CPA production by isolated strains from feed samples showed different percentages of CPA production reaching 80% in poultry feeds (Astoreca et al., 2011) and 61% in maize (Giorni et al., 2007).

On the basis of sclerotia size, *A. flavus* isolates can be divided into the S (<400 µm in diameter) and L (>400 µm in diameter) types. In our case, all sclerotia were of the L type. Similar results have been reported by other authors (Astoreca et al., 2011; Atehnkeng et al., 2008; Giorni et al., 2007; Perrone et al., 2014; Probst et al., 2014). Furthermore, in our study no correlation between sclerotia presence and toxigenicity was found; the non aflatoxigenic strains (chemotype IV and V) showed relatively hight sclerotia producers (60.53% and 55.65% respectively) than aflatoxigenic strains (chemotype I with 31.83%). These results corroborate with the findings of Giorni et al., 2007 and Rodrigues et al., 2009. In contrast, some studies reported a positive correlation between small sclerotia and aflatoxins production (Cotty, 1989; Pildain et al., 2004).

3.6 Aflatoxin B_1 contamination

The raw materials used in animal feed production are usually the source of aflatoxins. In this study, 37 out of 101 samples (36.6%) had a AFB_1 contamination level between 0.34 and 171.06 µg/kg and a median of 3.21 µg/kg (Table 6). No aflatoxins contaminations have been observed in wheat bran samples. Five samples of maize and one sample of pelleted cattle feed were found to contain AFB_1 above 20 µg/kg, exceeding the legal limit imposed by the Algerian (FAO, 2004) and European Union (EU) regulations (EU, 1881/2006). The most infected product was maize (75.8%) with 171.06 µg/kg.

Unacceptable levels of AFB_1 in animal feed samples have been reported by many authors. Lutfullah and Hussain (2012) showed a 40% aflatoxin contamination rate in maize, while in

China dairy feed showed 35.1% samples positive for AFB_1, with a mean contamination of 24.4 µg/kg (Xiong et al., 2018). In Cameroon, 9% of maize were contaminated with aflatoxins (from <2 to 42 µg/kg) (Kana et al., 2013). The study of Gizachew et al. (2016) conducted in Ethiopia showed different levels of aflatoxins in animal feeds. Out of 156 collected feed samples, 41 (26.2%) contained AFB_1 at a level exceeding 100 µg/kg.

Opposite results were found in Spain where low aflatoxins content was reported in dairy milk feedstuff samples with 85% were contaminated by AFB_1 and a mean concentration of 0.04 µg/kg. At the same time, none of the samples exceeded the statutory AFB_1 level of 5 µg/kg (Hernández-Martínez and Navarro-Blasco, 2015). Udom et al. (2012) reported 92% of dairy cattle feed not complying with the 5 µg/kg maximum limit for AFB_1 defined by the EU regulation (EU, 1881/2006). In a similar study conducted in Argentina, Greco et al. (2014) found that 44 out of 49 samples were contaminated with aflatoxins with a median of 2.68 µg/kg. The diversity of the AFB_1 contamination level can be due to the heterogeneity in pre-harvest climatic conditions and post-harvest management strategies developed in the different countries listed.The presence of mycobiota in a given commodity can be used as a guideline to estimate the potentially contaminating mycotoxins. Many factors such as substrate, pH, water activity and temperature affect the presence of aflatoxins in feeds during storage (Gonzales Pereyra et al., 2012). In our case, no correlation between the level counts of mycoflora and the AFB_1 concentration has been shown. This can be due to the fact that the main ingredients (maize and soybean) used in Algerian feed production are imported leading to a switch in the microbial population during transportation by boats (Bessaoud, 2019). Furthermore, mycotoxins are more resistant than fungal mycctelia to the processing chain of feed fabrication, they can be found in samples where the fungus can no longer be isolated (Omeiza et al., 2018).

4 Conclusion

According to our results, 98% of the animal feed samples were contaminated by fungi and Approximately half (49.01%) of *Aspergillus* section *Flavi* isolates were aflatoxigenic. AFB_1 was found in 36.6% (37 out of 101) samples and six samples exceeded the authorized limit (20 µg/kg).

In the Mediterranean region and particularly in Algeria, the climatic conditions are favorable for the development of *Aspergillus* species and the production of aflatoxins.

Besides, animal feed ingredients (maize, soybean…) are stored under natural conditions using traditional storage practices in open air and poor hygienic conditions. Therefore, continuous assessment of the mycoflora and aflatoxins in feeds should be implemented in order to ensure a safe consumption. The recognition of problems caused by mycotoxins in feeds is undoubtedly the first step toward the adoption of appropriate measures for the prevention and reduction of this problem, especially in a context where Algerian is constantly increasing their self-sufficiency in milk production.

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Table 1. Mean percentage of infection of the frequent genera isolated from maize and barley kernels using the direct plating method.

Substrate		**Infected kernels (%)**	***Aspergillus* (%)**	***Penicillium* (%)**	***Fusarium* (%)**	**Others (%)**
Raw materials	Maize (n=29)	86.77	58.77	25.04	06.61	09.10
	Barley (n=5)	78.17	73.17	8.17	05.50	12.66

Table 2. Fungal counts (CFU/g) obtained by the dilution method and the frequency of the main genera in raw materials and finished feed.

Substrate			**TFC (CFU/g)**	***Aspergillus* (%)**	***Penicillium* (%)**	***Fusarium* (%)**	**Others (%)**
Raw materials	Maize (n=29)		$4.7\times10^4 \pm 2.2\times10^{4\ b}$	02.87	05.45	91.65	00.02
	Barley (n=5)		$7.7\times10^3 \pm 5.6\times10^{3\ a,b}$	21.74	17.39	60.87	00.00
	Soybean (n=10)		$5.9\times10^3 \pm 4.6\times10^{3\ a}$	31.09	15.63	52.94	00.28
	Wheat bran (n=7)		$2.4\times10^4 \pm 1.3\times10^{4\ a,b,c}$	69.64	20.24	06.55	02.94
Finished feed	Poultry feed	Ground (n=22)	$4.5\times10^4 \pm 4.4\times10^{4\ b}$	10.43	22.36	64.21	03.01
		Pelleted (n=12)	$2.5\times10^2 \pm 2.3\times10^{2\ a}$	50.00	23.33	26.67	00.00
	Cattle feed	Ground (n=7)	$3.3\times10^4 \pm 1.7\times10^{4\ b,c}$	08.52	30.72	60.75	00.00
		Pelleted (n=9)	$8.0\times10^2 \pm 6.8\times10^{2\ a}$	58.93	28.57	12.50	00.00

Same letters in a column are not significantly different at $p \leq 0.05$. TFC: Total Fungal Counts, CFU/g: Colony Forming Units per gram.

Table 3. Occurrence and abundance of *Aspergillus* in raw materials and finished feed.

Substrate			***A. Flavi* (%)**	***A. Terei* (%)**	***A. Nigri* (%)**	***A. Clavati* (%)**	***A. Candidi* (%)**	***A. Fumigati* (%)**
Raw materials	Maize	DM	59.02	08.20	-	-	-	32.79
		DP	63.43	00.48	10.17	05.09	-	20.83
	Barley	DM	80.00	-	20.00	-	-	-
		DP	87.05	-	03.60	-	-	09.35
	Soybean		79.46	01.08	03.24	16.22	-	-
	Wheat bran		59.83	37.61	00.85	-	00.85	00.85
Finished feed	Poultry feed	Ground	50.00	-	42.16	-	07.84	-
		Pelleted	86.67	06.67	06.67	-	-	-
	Cattle feed	Ground	74.62	09.64	-	-	04.06	11.67
		Pelleted	68.08	19.15	02.13	-	08.51	02.13
Mean percentage (%)			70.81	08.28	08.88	02.33	02.13	07.75

DM: Dilution Method, DP: Direct Plating method.

Table 4. Toxigenic potential of *Aspergillus* section *Flavi* isolates.

Substrat			**Tested strains**	**Number of toxigenic strains**	**Percentage (%) of toxigenic strains**
Raw materials	Maize		203	124	61.00
	Barley		76	26	34.20
	Soybean		65	20	30.70
	Wheat bran		39	18	46.15
Finished feed	Poultry feed	Ground	25	15	60.00
		Pelleted	18	09	50.00
	Cattle feed	Ground	20	10	50.00
		Pelleted	13	03	23.00
Total			459	225	49.00

Table 5. Incidence of *A. flavus* chemotypes (AFs and CPA production) and sclerotia presence and type.

Morphotypes	Chemotypes	Number (%) of each chemotype	Toxigenicity [a]			Number (%) of sclerotia producers	Type of sclerotia [b]
			AFB	AFG	CPA		
A. flavus	I	223 (48.58)	+/++	-	+	71 (31.83)	+
						152 (68.16)	-
	II	00 (00.00)	+	+	+	00 (00)	-
	III	00 (00.00)	+	-	-	00 (00)	-
	IV	114 (24.84)	-	-	+	69 (60.53)	+
						45 (39.47)	-
	V	115 (25.05)	-	-	-	64 (55.65)	+
						51 (44.35)	-
A. parasiticus	VI	02 (00.44)	+	++	-	00 (00)	-
A. tamarii	IV	05 (01.09)	-	-	+	00 (00)	-
Total (%)		459 (100)	225 (49.01)	2 (00.44)	342 (74.5)	204 (44.44)	

[a] ++ high intensity signal; +: medium intensity signal; +/−: low signal; -: not detected. [b]-: no sclerotia observed; + presence of large sclerotia (L); AFs: aflatoxins; AFB: aflatoxins B; AFG: aflatoxins G; CPA: cyclopiazonic acid.

Table 6. Incidence and range of AFB_1 in raw materials and finished feed.

Substrate			Number of positive samples	Percentage (%) of positive samples	Range (µg/kg)	< 20 µg/kg		≥20 µg/kg	
						Number	Range (µg/kg)	Number	Range (µg/kg)
Raw materials	Maize		22	75.80	00.34-171.06	17	00.34 -16.50	5	23.85-171.06
	Barley		03	60.00	00.78-01.60	3	00.78-01.60	00	
	Soybean		02	20.00	00.59-00.60	2	00.59-00.60	00	
	Wheat bran		00	00.00	00	00	00	00	
Finished feed	Poultry feed	Ground	03	13.63	00.79-02.20	3	00.79-02.20	00	
		Pelleted	04	33.33	00.43-01.05	4	00.43-01.05	00	
	Cattle feed	Ground	01	14.30	01.31	1	01.31	00	
		Pelleted	02	22.22	02.02-23.60	1	02.02	1	23.60
Total (n=101)			37	36.6	00.34-171.06	31	00.34 -16.50	6	23.85-171.06

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Incidence of mycobiota and aflatoxin B1 in Algerian feed

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