An - Najah National University
Faculty of Graduate Studies

A Microbiological Study on Poultry Feed with Special Emphasis on Aflatoxigenic Aspergillus Flavus

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biological Science, Faculty of Graduate Studies, at An- Najah National University, Nablus, Palestine.

March 2000

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TO

MY DEAR MOTHER, HUSBAND,BROTHERS,

AND SISTERS FOR THEIR SUPPORTS AND

ENCOURAGEMENT, WITH LOVE AND RESPECT

ACKNOWLEDGMENT

I wish to express my appreciation and gratitude to my supervisor Prof. Mohammed S. Ali-Shtayeh for guidance and help throughout this work. My thanks also go to my co-supervisors Dr. Yahya R. Faydi and Dr. Jamal Abu-Omer for their valuable suggestions and comments during this research.

I would also like to thank Mr. Esmail EL-Masri of the Scientific Center for Food and Drug Analysis in Nablus, for technical help with aflatoxin analysis.

My thanks are also due to Mr. Raid Esefan, my colleagues: Rana Jamous, Lubna EL-Kharraz and Rafeef EL-Khateeb, for their support and encouragement throughout this work.

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Abstract

A total of 100 samples of mixed poultry feeds and component raw materials including 28 mixed poultry feed, 25 yellow corn, 25 soybeans, 14 premix, and 8 sorghum were collected randomly from eight feed factories from the West Bank, over a 12-month period. The samples were assayed for their microbiota including *Aspergillus flavus* and *Salmonella* using standard procedures. The incidence of detection aflatoxigenic *Aspergillus flavus* was determined using coconut agar medium (CAM) and U.V light. Aflatoxins were determined in poultry feed samples with aflatoxigenic *A. flavus* using thin-layer chromatography (TLC) technique. All 100 samples of mixed poultry feeds and component raw materials were negative for *Salmonella.* Soybeans and yellow corn had the highest total aerobic bacteria (CFU g^{-1} Dwt.) ($6.3x10^5$ and $3.11x10^5$, respectively) and total mold count (CFU g^{-1} Dwt.) (47640 and 73540, respectively). This could be attributed to high moisture content and high protein content and an elevated level of carbohydrates and/ or lipids in these feed components. Premix samples had the lowest total mold count (2489.28) probably due to low moisture content. For each sample of mixed poultry feeds and component raw materials in the present work, total mold count was found to be higher in the cold season than in the warm season. This was attributed to higher moisture content during the rainy cold period. Of the 100 samples studied 15 species belonging to 5 genera of filamentous fungi were identified. *Aspergillus* species (95%) and *Fusarium* (68%) were the most dominant fungi isolated from mixed poultry feeds samples. Eleven species of *Aspergillus* were identified

including *A. restrictus*, *A. glaucus*, *A. candidus and A. flavus*. A total of 114 isolates of *A. flavus* were recovered from 35 poultry feed samples. 64 (56%) of these isolates were found to be aflatoxigenic. TLC analysis revealed that 4 out of the 35 samples contained aflatoxins. The level of aflatoxins B_2 and G_2 in yellow corn and mixed poultry feeds ($\leqslant$ 5 ng g^{-1}) did not exceed the standard levels ($<$ 20 ng g^{-1}).

CHAPTER ONE

GENERAL INTRODUCTION

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Contaminated animal feeds can pose a potential and/or real health threat, due to the possible carry-over of some fungi toxic residues mycotoxins into the animal products and thus into the food chain (Smith *et al.*, 1983). Contamination of animal feeds decreases their nutritive value and affect animal and human health (Herry *et al.*, 1987; Bauduret, 1988; Bauduret, 1990). Surveys are therefore, usually done regularly in order to obtain data on fungal and bacterial occurance in raw materials and mixed feeds (Herry *et al.*, 1987; Bauduret, 1988). Even though, in developed countries, heavily contaminated food supplies are not permitted in the market, concern still remains for possible adverse effects resulting from long-term exposure to low level of such toxins in the food supply (Finley *et al.*, 1992).

1.1 Mycotoxins

Mycotoxins: are chemical compounds produced by fungi growing on organic substances such as corn, cotton seed or peanuts, which have some undesirable effects to the animal consuming them. These effects range from vomiting, feed refusal, and weight loss, to various types of

tumors, and, in some cases, death (Liener, 1969; Wyllie & Morchause, 1978). More than 100 toxic compounds produced by fungi have been identified and about 45 of these occur in grain crops (Wyllie & Morchause, 1978). Some mycotoxins are rather rare in occurance; others such as aflatoxins are quite common in some years (Wyllie & Morchause, 1978). The mycotoxin was named aflatoxin, the "a" from *Aspergillus* and "fla" from *flavus* (Heathcote & Hibbert, 1978).

Mycotoxins have probably been present in man's food supply since the beginning of civilization (Wyllie & Morchause, 1978). However, increased use of mechanical harvesting may have resulted in increased harvest of badly molded grains that would have been discarded with hand harvesting (Wyllie & Morchause, 1978; Smith & Moss, 1985).

1.2 Aflatoxins

Aflatoxins: are a group of structurally related toxic compounds discovered in 1960, after a severe toxic outbreak among farm animals in England, which became known as "Turkey X Disease" because of the involvement of large numbers of turkey poults (Burnside *et al.*, 1957; Edlefsen & Brewer, 1996; Moss, 1996). These toxins are most commonly produced by strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Goldbatt, 1969; Wyllie & Morchause, 1978; Norton, 1997).

These fungal secondary metabolites are formed by consecutive series of enzyme catalyzed reactions from a few biochemical simple intermediates of primary metabolism *e.g.*, acetate, mevalonate, malonate and certain amino acids. The main biosynthetic reaction includes alkylation, halogination, condensation and oxidation / reduction (Smith *et al.*, 1983; Steyn, 1998).

A suitable substrate is required for fungal growth and subsequent toxin production (Smith *et al.*, 1983; Finley *et al.*, 1992; Atkin, 1997). Water stress, humidity, high temperature stress, and insect damage of the host plant are major determining factors in mold infestation and toxic production. Similarly, specific crop growth stage, poor fertility, high crop densities and weed competition have been associated with increased mold growth and toxin production. Aflatoxin formation is also affected by associated growth of other molds or microbes (Smith *et al.*, 1983; Finley *et al.*, 1992; Moss, 1996; Atkin, 1997).

Cereals can be highly susceptible to fungal growth when still in the field, during storage and in processing (Smith *et al.*, 1983). But detectable amounts of aflatoxins do not usually occur, even in the aflatoxin presence of isolates of *Aspergillus* species (Bean *et al.*, 1972; Smith *et al.*, 1983).

The major aflatoxins of concern are designated B_1, B_2, G_1, G_2 and M_1. Molecular formulas established from elementary analysis and mass

spectrometric determination for main aflatoxins are as follows (Smith & Moss, 1985; Finley *et al.*, 1992).

B_1: $C_{17}H_{12}O_6$ B_2: $C_{17}H_{14}O_6$

G_1: $C_{17}H_{12}O_7$ G_2: $C_{17}H_{14}O_7$

Aflatoxins B_2 and G_2 were established as the dihydroxy derivatives of B_1, and G_1, respectively. Whereas, aflatoxin M_1, is hydroxy aflatoxin B_1, and aflatoxin M_2 is 4- hydroxy aflatoxin B_2 (Finley *et al.*, 1992).

The aflatoxin M was first isolated from milk of lactating animals fed aflatoxin preparations; hence, the M designation. Whereas the B designation of aflatoxin B_1 and B_2 resulted from the exhibition of blue fluorescence under UV-light, while the G designation is referred to the yellow-green fluorescence of relevant structures under UV-light (Heathcote & Hibbert, 1978; Eaton & Groopman, 1994; Doyle *et al.*, 1997). Aflatoxins B_1, B_2, G_1 and G_2 were identified according to their blue or green fluorescence properties and migration patterns during chromatography (Heathcote & Hibbert, 1978). When a commodity of aflatoxins is analyzed by thin-layer chromatography, the aflatoxins separate into the individual components in the order given above.

However, the first 2 (aflatoxin B_1, aflatoxin B_2) fluoresce blue when viewed under ultraviolet light and the other two aflatoxins (G_1, G_2) fluoresce green (Goldbatt, 1969).

In 1960 only aflatoxin B_1 was legislated for in feed, because it constituted a high health risk particularly because of its known carcinogenicity (Smith *et al.*, 1983). Legal standard for aflatoxin B_1 in the whole feedstuffs for poultry and pigs is 0.02 μg/kg (Smith *et al.*, 1983).

Many countries have set legislative limits to the concentrations of a number of mycotoxins in food, and these may reflect analytical capability rather than a clear understanding of the toxicological significance or distribution of mycotoxins in food (Table 1.1)(Pohland, 1993).

Table 1.1 Range of regulatory limits for mycotoxins*.

Mycotoxin	Registered limit (μg kg^{-1})	Number of countries
Aflatoxin in food	0-50	53
Aflatoxins M_1 in milk	0-0.5	15
Chaetomin	0	1
Deoxynivalenol	1000-4000	5
Ochratoxin	1-300	6
Patulin in apple juice	20-50	10
Phomopsin	5	1
Stachyobotryotoxin	0	1
T-2 toxin	100	2
Zearalenone	30-1000	4

* After: Pohland (1993).

The Food and Drug Administration (FDA) in1965, set limits for these toxins in foods and feeds involved in interstate commerce (Smith *et al.*, 1983). The total level of aflatoxin B_1, B_2, G_1, and G_2 in any food or feed should not exceed 20 μcg kg^{-1} (20 ppb) or 300 μcg kg^{-1} (300 ppb) for cotton seed meal. The total level of aflatoxin M_1 allowed in milk is 0.5 μcg kg^{-1} (Edlefsen & Brewer, 1996). Even with such data it is not easy to define a level of acceptability (Smith *et al.*, 1983).

Table 1.2 Regulation of total aflatoxins levels for ruminants according to the Food and Drug Administration (FDA)*.

Feed stuff	Aflatoxin level (ppb)
Corn used in dairy animals and immature animals rations	<20
Corn used by breeding cattle	<100
Corn used by finishing beef cattle	<300
All feed stuffs other than corn	20
Cotton seed meal as a feed ingredient	300

*After: Eaton & Groopman (1994).

1.3 The Fungus

1.3.1 *Aspergillus flavus*

Aspergillus flavus Link ex Fries is actually not a single species, but a "species complex " made up of eleven species that are known to occur in many kinds of plant materials, including stored grains, producing aflatoxins when moisture content and temperature range are optimal for their production (Liener, 1969; Smith *et al.*, 1983). The amount of aflatoxin produced differs according to the substrate on which *A. flavus* is growing, although the mycelial mass may be the same (Liener, 1969;

Smith *et al.*, 1983). *A. flavus* does not normally contaminate grains and other crops while they are in the field. It is only after the grains are harvested and stored they may become contaminated with the fungus (Smith & Moss, 1985; Finley *et al.*, 1992). Also *Aspergillus* species have recently become a major cause of invasive fungal disease in immunocompromised host (Vazquez *et al.*, 1998).

1.3.2 Conditions favoring growth of *A. flavus*

The occurrence of aflatoxins varies with climatic conditions prevailing at time of harvest and transportation and during later conditions (Smith *et al.*, 1983; Neal, 1987). Moisture level of grains at harvest is an important determining factor to subsequent mold colonization (Smith *et al.*, 1983; Finley *et al.*, 1992).

High moisture levels (20-25% wet weight) are required for the growth of fungi in the living plant or in decaying organic material, whereas fungi capable of growing in stored products can usually exist on moisture content of (12-18%) (Smith & Moss, 1985) (see also Table 1.3).

In many cases, development of the fungus usually stops when the temperature is below -1.5 °C and grain moisture is 12 percent or below.

Table 1.3 Conditions favoring the aflatoxin-producing fungus, *A. flavus* growing on corn kernels *.

Factor	Optimum	Range
Temperature (°F)	>68	80 to 200
Relative humidity (%)	>85	62 to 99
Kernel moisture (%)	18	13 to 20

*After: Wyllie & Morchause (1978).

1.4 Effects of aflatoxins on human and animal health

Aflatoxins have been associated with various diseases, such as aflatoxicosis in livestock, domestic animals and humans throughout the world (Finley *et al.*, 1992). The fact that so many animals species are susceptible to aflatoxin, showing acute and chronic symptoms, must be considered as strong evidence that man could well be similarly affected if submitted to similar exposure levels (Smith *et al.*, 1983).

Aflatoxins, which cause aflatoxicosis in some animal species, were also potent hepatocarcinogens (Goldbatt, 1969; Smith & Moss, 1985; Finley *et al.*, 1992). Aflatoxicosis is primarily a hepatic disease. The susceptibility of individual animal to aflatoxins varies considerably depending on age, sex, animal species and nutrition (Smith *et al.*, 1983; Finley *et al.*, 1992). Small amount of aflatoxin causes liver damage,

decreases milk and egg production, recurrent infection as a result of immunity suppression (Finley *et al.*, 1992; Cotty, 1997).

Low concentration of aflatoxin was found to induce immunologic deficiency in animals and considered to be associated with the specific failure of the cell-mediated immune system to function, while at somewhat higher levels was found to impair antibody production (Oswald & Comera, 1998; Smith *et al.*, 1983; Sharman *et al.*, 1992). In addition to embryo toxicity in animals consuming low dietray concentration, clinical stage of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproductivity, reduced feed utilization and effeciency, anemia and jaundice (Goldbatt, 1969; Liener, 1969; Finley *et al.*, 1992).

The induction of cancer by aflatoxin is extensively studied (Goldbatt, 1969). In fact, B_1, M_1 and G_1 aflatoxins were found to cause various types of cancer in different animal species (Goldbatt, 1969). However only aflatoxin B_1 is considered by International Agency for Research on Cancer [I A R C] as having produced sufficient evidence of carcinogenicity in experimental animals (Goldbatt, 1969; Finley *et al.*, 1992; Edlefses & Brewer, 1996). B_1, G and M were found to inhibit oxygen uptake in whole tissue homogenates from several animal species (Goldbatt, 1969). Aflatoxin requires metabolic activation to exert its

carcinogenic effects, and these effects can be modified by induction or inhibition of the mixed function oxidase system (Goldbatt, 1969).

There has been some evidence of an increased incidence of cancer in workers exposed for, 2 - 9 years, to dust from oil seed crushing mill which handled groundnuts containing 0.87 – 72 ng aflatoxin per cubic meter of air with a corresponding respiratory exposure of 39 ng to 3.2 µg per worker per week (Smith and Moss, 1983).

1.5 *Salmonella:*

1.5.1 Genus *Salmonella*

Salmonella species are facultative anerobic gram-negative rods belonging to the family Enterobacteriacae. Although members of this genus are motile by peritrichous flagella, non-flagellated variant such as *Salmonella pullorum* and *Salmonella gallinarum*, are non- motile strains resulting from dysfunctional flagella do occur (Doyle *et al.*, 1997).

Compared with other gram-negative rods, *Salmonella* is relatively resistant to various environmental factors; it grows at temperatures between 8 and 45°C and a pH range of 4 – 8, and it is sensitive to heat and easily killed by pasteurization (WHO, 1988; Doyle *et al.*, 1997). Typical *Salmonella* isolate would produce acid and gas from glucose in triple sugar iron agar (TSI) (WHO, 1988; Baron *et al.*, 1994). *Salmonella*

are oxidase negative and catalase positive, catabolize D-glucose and other carbohydrates with the production of acid or acid and gas, grow on citrate as the sole carbon source, generally produce hydrogen sulfide, decarboxylate lysine, and ornithine and do not hydrolyze urea (Doyle *et al.*, 1997). *Salemonella* can withstand drying for years, especially in dried factors, and other dry materials such as animal feeds and certain foods (WHO, 1988).

The prevailing hygienic condition during the production, harvesting, and distribution of agricultural food do not always meet minimum standard, which facilitate product contamination (Doyle *et al.*, 1997). The importance of sanitory practice during the harvesting, processing, and distribution of raw food and food ingredient is very important to minimize the incidence of salmonellosis (WHO, 1988).

1.5.2 Animal feed as a reservoir of *Salmonella*

Contaminated animal feeds are considered as potential sources from which farm animals may acquire *Salmonella* (Roberts, 1990). Previous reports from various countries have indicated that animal feeds are more or less contaminated with *Salmonella* (Roberts, 1990). In addition to the risk of clinical salmonellosis in animals ingesting

contaminated feed, human subjects may be infected as a result of consuming infected meat (Roberts, 1990).

Research shows that feeds of animal origin frequently are sources of *Salmonella* serotypes (Morgan et al., 1985; ESH, 1994). Surveys of 1,679 samples of animal byproduct collected from feed mills in Minnesota showed a contamination rate of 20% and 35 different serotypes (ESH, 1994).

Decontamination of animal feed through irradiation has a number of advantages, including the fact that it can be performed after bagging of feed, thus averting recontamination (ESH, 1994). So that much of the *Salmonella* infection of animals could be prevented by environmental hygiene, by elimination of the organism from animal protein feeds, by proper cleanup of animal-rearing facilities and holding areas, and by prevention of contamination during transport to slaughter (ESH, 1994).

1.5.3 Pathogenicity of *Salmonella*

Salmonella is often pathogenic to humans or animals when acquired by the oral route (Van Poucke, 1990). *Salmonella* infection results from the ingestion of food or water containing sufficient numbers of these bacteria to reach and invade the small intestine. Clinical symptoms include acute gastroenteritis, bacteremia with or without

localized extra-intestinal infection, typhoid-like fever, and an asymptomatic carrier state (Van Poucke, 1990; Gillespie, 1994). *Salmonella* infection is responsible for 10 to 15% of acute gastroenteritis cases (Van Poucke, 1990). In recent years, it has been found that consumption of food containing as low as 3 - 10 cells per gram may cause disease (WHO, 1988), and symptoms of salmonellosis occur 12 - 24 hours after ingestion of the contaminated food (Hui, 1990).

1.6 Objectives:

The present work was aimed at:

1. Investigating mycobiota including aflatoxigenic *Aspergillus flavus* of mixed poultry feeds and their components.
2. Studying the prevalence of *Salmonella* in mixed poultry feeds and their components.
3. Identification and quantitation of aflatoxins in aflatoxigenic *Aspergillus flavus* contaminated feed.
4. Assessing the role of animal feeds and components in the epidemiology of mycotoxins and *Salmonella.*

CHAPTER TWO

MATERIALS &METHODS

CHAPTER TWO

MATERIALS AND METHODS

2.1 Sample collection

A total of 100 1 kg-samples of mixed poultry feed and component raw materials (mixed poultry feeds, 28; yellow corn, 25; sorghum, 8; soybeans, 25; and premix, 14), were collected randomly from eight feed factories from the West Bank, over a 12- month period during warm and cold seasons, between July 1997 and June 1998.

The samples were collected monthly, taken back to the laboratory in labeled plastic bags and were stored at 4 °C until processed.

2.2 Processing and culturing of samples

As soon as samples arrived at the laboratory, each one was assigned an individual unit number. Examination of mycobiota, total aerobic bacteria and *Salmonella* for each sample, were carried out within 48 hours of collection.

2.3 Determination of moisture content

Samples were ground and the moisture content of each sample was determined by weighing two 20 g aliquots and dried in an oven at 80

°C for 16 hours. The aliquots were then weighed and the initial water content percentage was determined (Herry *et al.*, 1987; Dalcero *et al.*, 1997).

2.4 Mycological studies (mycobiota determination)

2.4.1 External mycobiota

External mycobiota was determined by placing 20 g sub samples in 180 ml of solution A (Appendix A), and mixed throughly on an orbital decimal shaker (400 rpm) for 20 minutes (Bauduret, 1988).

Ten fold dilutions from the previous solution mixture were prepared in 1% peptone-water solution, one ml solution was added to 9 ml 1% peptone-water solution to give 10^{-2} dilution. This step was repeated to give the appropriate decimal dilution $10^{-3} - 10^{-4}$. From each decimal dilution one ml was pipetted onto 4 plates of each of M2 and M5 agar media (Appendix A), containing 60 µg/ml chloramphenicol and 50 µg/ml gentamycine sulfate (Wicklow *et al.*, 1981; Bauduret, 1990).

Of each media, two of the inoculated plates were incubated at 25 °C and the other two at 35 °C for 4-6 days (Diener & Davis, 1966; Bauduret, 1988), as the optimum temperature for *A. flavus* growth occurs at 35 °C and the maximum aflatoxins biosynthesis occurs at 25 - 30 °C on both synthetic and natural media (Smith & Moss, 1983; Van Egmond,

1989). The plates were observed daily and only plates that contained 10 - 100 colonies were considered to count as colony forming units (CFU) gm^{-1} dry weight (Dwt.) of sample. *A. flavus* colonies were also counted and results recorded as CFU gm^{-1}

2.4.2 Internal mycobiota

Internal mycobiota was determined only on samples of whole corn. From each sample, 50 whole kernels were surface-sterilized for 1 - 2 min with sodium hypochlorite solution containing about 1000 p.p.m available chlorine, and washed twice with sterile distilled water (Mills *et al.*, 1996; Ramirez *et al.*, 1996)

Twenty five kernels were plated on M2 agar medium, and 25 kernels on M5 agar medium (five kernels per plate). After 7 days of incubation at 25 °C, the infecting or internal fungi were counted and identified (Bauduret, 1988; Gonzalez *et al.*, 1996). Results were expressed as percentage of mold-infected kernels.

2.4.3 Identification of fungi

Growing fungal colonies were identified by subculturing on potato-dextrose agar medium (PDA) (Appendix A). Taxonomic identification of all colonies was achieved through macroscopic and microscopic examination using lactophenol-aniline blue stain, followed

by standard tests related to the genera of each particular group of fungi (Dalcero *et al.*, 1998), and with the aid of published taxonomic keys and monographs (Lin & Dianese, 1976; Davis *et al.*, 1987).

After grouping *A. flavus* colonies growing in each plate on the basis of their macroscopic features (*e.g* color), one colony of each group was selected for qualitative test (Lin & Dianese, 1976).

2.5 Detection of aflatoxins

2.5.1 Qualitative Analysis

A qualitative test for aflatoxin-producing strains of *A. flavus* was carried out by culturing *A. flavus* isolates on coconut agar medium (CAM) (Appendix A). A mycelial plug from PDA was placed onto the center of CAM plate and the plate was incubated at 25 °C for a period of up to one-week (Lin & Dianese, 1976).

Plates were examined daily under U.V. lamp at 365 nm in a dark room, until the seventh day of incubation, for presence or absence of blue fluorescence in the agar surrounding the colonies (Bauduret, 1988). The intensity of fluorescence expressed by number of "+" signs (Lin & Dianese, 1976).

Yellow-orange pigmentation was observed seen before the appearance of fluorescence, and the degree of pigmentation seemed to be

proportional to the intensity of fluorescence. All of the aflatoxin- positive fluoresced, but none of the aflatoxin-negative isolates, produced the yellow-orange pigmentation (Lin & Dianese, 1976; Herry *et al.*, 1987). Samples containing fluorescence isolates was kept on sabouraud's dextrose agar (SDA) medium (Appendix A) at 4 °C for mycotoxin analysis (Koneman & Roberts, 1985).

2.5.2 Quantitative analysis

Since not all isolates of *A. flavus* can produce aflatoxins, determination of aflatoxin-producing ability of a particular isolate is important in biochemical, genetical, toxicological, and epidemiological studies. The method commonly used to assay for aflatoxin production is to inoculate various liquids or solid substrates with the fungus and to incubate for a period of time. Aflatoxins then are extracted from the substrates with organic solvents, lipid removal, cleanup, concentrated, separated and assayed by thin-layer chromatography (TLC) (Davis *et al.*, 1966; Lin & Dianese, 1976).

Depending on the nature of the commodity, TLC method can sometimes be modified by omitting unnecessary steps (Goldbatt, 1969). It is an official method in which aflatoxins can be identified and quantified

at levels as low as 1 ng / g. This is however a time-consuming and laborious procedure (Davis *et al.*, 1966; Lin & Dianese, 1976).

Analysis of aflatoxins was carried out using methods described by Smith & Moss. (1985) and Waliyar *et al.* (1994):

1. One hundred gram of each sample was ground and a 25-gram sub-sample was used for extraction in 50 - ml aqueous methanol solution (80 % v / v).
2. 1 ml of filtrated extract was diluted with 4 ml of 0.5 % acetic acid and passed through pre-packed cyanide (CN) column (0.5 gm) after conditioning it with 2 ml (0.5 %) aqueous acetic acid.
3. The column was defatted by washing with 2-ml n-hexane and air dried for 3 minutes.
4. Defatted residue (*e.g* aflatoxins) was eluted from the column with 2-ml methylene chloride.
5. Methylene chloride was reconstituted with 100 µl of methanol and then 100 µl was applied to disc, evaporated, and concentrated to dryness.
6. The disc was plotted on TLC plate. Different standard aflatoxin references of (B_1, B_2, G_1, and G_2) were used on the same plate.
7. TLC plate was developed in toluene - ethylacetate - formic acid (60 %, 32 %, and 8 %, respectively).

8. TLC plate was examined under UV light at 365 nm in dark room, and the diameter of any green or blue fluorescence was measured and recorded. Diameters of standard aflatoxins were also measured and recorded.

Aflatoxins concentrations was determine as follows:

$$\frac{\text{Diameter of the sample}}{\text{Diameter of standard} \times 25\ \text{gm}} \times \text{Concentration of standard}$$

2.6 Bacteriological studies

2.6.1 Processing and culturing of samples

A. Pre-enrichment media

Twenty grams of each assigned sample were inoculated aseptically into 500 - ml flask containing 180 ml of sterile nutrient broth. After mixing by swirling, the flask was incubated at 35 °C for 24 hours.

B. Total aerobic bacteria count

Total aerobic bacterial count was determined following method described by Herry *et al.* (1987):

1. Ten fold dilutions from the previous broth mixture were prepared in 1% peptone-water solution, one ml broth was added to 9 ml 1% peptone-water to give 10^{-2} dilution. This step was repeated to give the appropriate decimal dilution $10^{-3} - 10^{-6}$.
2. From each decimal dilution, 1 ml was transferred to an empty sterile plate. Two plates were used for each dilution.
3. Each plate was then over layed with 15 – 20 ml of plate count agar (PCA) (Appendix A) and mixed throughly.
4. After solidification, the plates were incubated at 35 °C for 18 - 24 hours in an inverted position.

5. The plates that contained 15-150 CUF were considered for count, and the results were expressed as CFU gm^{-1} of samples (Marshall, 1992).

The average colony count for 2 plates of each sample was multiplied by the dilution factor to determine the CFU gm^{-1}.

3.7 Isolation of *Salmonella*

Isolation of *Salmonella* was carried out using method described by Herry *et al.* (1987) as follows:

3.7.1 Selective enrichment

1. After incubation, 1 ml from the previous nutrient broth mixture was transferred aseptically to 10 ml selenite cystine and another 1 ml to 10 ml tetrathionate broth.

2. Both cultures were incubated at 35 °C for 24 hours (Herry *et al.*, 1987).

3.7.2 Selective growth

1. After incubation of tetrathionate and selenite cystine broth, the tubes were mixed by using a vortex.

2. Using a sterile loop, 3mm loopfull were taken aseptically from each culture and streaked on xylose lysine deoxycholate agar (XLD) and brilliant green agar (BGA).

3. The plates were incubated at 35 °C for 24 hours.

4. In the following day, the plates were examined for the presence of *Salmonella* suspected colonies.

On (BGA), *Salmonella* appeared as red-pink-white opaque colonies surrounded by brilliant red zones in the agar while on (XLD) agar, *Salmonella* appeared as pink-red colonies with or without black centers.

3.7.3 Screening:

1. Three colonies typical or suspected to be *Salmonella* were selected from each selective agar for further identification.
2. The very center of the colony to be picked was lightly touched with sterile inoculating needle and TSI agar slant was aseptically inoculated by streaking slant and stabbing butt.
3. The TSI agar slant was incubated for 24 hours at 35 °C with loose cap to maintain aerobic condition while incubating slant and to prevent excessive H_2S production.

Cultures, which produced alkaline (red) slant and acid (yellow) butt with or without production of H_2S (blacking) in TSI Agar, were retained as potential *Salmonella* isolates and were submitted for other biochemical and serological tests.

3.8 Identification of *Salmonella*

A. Urease test (conventional) (Baron *et al.*, 1994)

1. The suspected colony was lightly touched with a sterile inoculating needle, and aseptically inoculated onto urea agar slant by streaking.
2. The tube was incubated at 35 °C for 24 hours.

All cultures that gave negative urease test (no change in color of medium) were retained for further identification, whereas all cultures that gave positive urease test (pink color) were discarded.

B. Lysine decarboxylase broth test (Andrew, 1985)

1. With a sterile needle, a small amount of growth from TSI agar slant suspicious for *Salmonella* was aseptically inoculated into lysine decaboxylase broth.
2. The cap was replaced tightly and the tube was incubated for 24 hours at 35 °C.

Cultures that caused alkaline reaction indicated by purple color throughout medium were considered as suspected *Salmonella* and were retained for further identification, whereas cultures showed development of yellow color throughout medium were discarded.

C. S.I.M. test

S.I.M. test was carried out following steps described by Baron *et al.* (1994):

1. A tube of S.I.M. medium was aseptically inoculated with a portion of bacteria colony by stabbing the media with a sterile inoculating needle once to a depth of 1/2 - 1/4 inch.

2. The tube was incubated overnight at 35 °C.

Reactions on S.I.M.

S: Blackening of tube – production of sulfide (sulfide positive);no change in the color of tube – sulfide negative. M: Brush like growth around line of inoculation or cloudiness throughout medium – positive motility (Baure, 1982). I: production of indole in such medium (Baron *et al.*, 1994; Baure, 1982), the latter was achieved by:

1.Adding 1 ml of xylene to 24-hour culture of organisms in SIM medium.

2. The tube was shaken well and allowed to stand for a few minutes until the solvent risen to the surface.

3. About 0.5 ml of Ehrlich reagent was gently added down the sides of the tube.

A distinct brilliant red ring, developed just below the solvent layer, represented a positive test while absence of such red ring represented a negative test. All *Salmonellae* are indole negative as indicated by absence of a brilliant red ring after the addition of Ehrlich reagent.

Most *Salmonellae* are motile organisms as indicated by producing cloudiness in the medium, or growing in brush-like patterns around the line of inoculation; while some *Salmonella* species produce hydrogen sulphide as indicated by blackening of the line of inoculation whereas others can not (Baron *et al.*, 1994).

Under aseptic condition, growth on the TSI agar slant, biochemicaly identified as *Salmonella* was inoculated into sterile nutrient agar slant by streaking the slant with a sterile inoculating needle. The slant was incubated at 35 °C for 24 hours.

From fresh nutrient agar slant culture, oxidase, catalase and serological tests were done.

D. Serological test

By using *Salmonella* polyvalent somatic (O) antiserum, serological test was carried out as follows:

1. The reagents were brought to room temperature, then mixed by vigorous shaking.
2. A portion of suspected bacterial colony was picked off aseptically, by using a sterile loop from fresh nutrient agar slant culture and then emulsified carefully in a saline drop on a clean slide.
3. One drop of antiserum was added to the suspension and mixed together with a sterile loop to spread and cover a reaction area.
4. The loop was flamed and the slide was rocked gently in a circular motion for only 1 minute.

Negative reaction was indicated by the absence of agglutination whereas positive reaction was indicated by agglutination with the test reagent within 1 minute, compared to the control.

CHAPTER THREE

RESULTS

CHAPTER THREE

RESULTS

3.1 Moisture content

Based on internationally acceptable moisture content levels (<12.0 %) of mixed poultry feeds and raw materials, the current work showed that soybeans and yellow corn had acceptable percentages (11.9 and 11.7 %, respectively; Table 3.1). Premix had a lower moisture content level (6.8 %). The moisture content of all the samples did not show any noteworthy variations (Appendix B).

3.2 Bacteriological analysis

A. Total aerobic bacteria

Soybeans and yellow corn gave the highest average total aerobic bacterial counts (CFU g^{-1} Dwt.) ($6.3x10^5$ and $3.11x10^5$, respectively), whereas mixed poultry feeds gave the lowest average total aerobic bacterial count ($0.93x10^5$; Table 3.1).

B. *Salmonella*

All 100 samples of soybeans, mixed poultry feeds, premix, sorghum and yellow corn were negative for *Salmonella.*

3.3 Mycological analysis

3.3.1 External mycobiota

Table 3.2 shows that all samples on M2 and M5 media had higher total mold count at 25 °C than at 35 °C except for sorghum, and generally all samples on M5 media had greater total mold counts than on M2 media. Yellow corn and soybeans total mold counts on M2, M5 media were the highest at both 25 °C and 35 °C, while sorghum and premix total mold counts on M2, M5 media were the lowest at both 25 °C and 35 °C

A. Total mold count

Yellow corn and soybeans gave highest average total mold counts (CFU g^{-1} Dwt.) (73540 and 47640, respectively) followed by mixed poultry feeds (30696.43), whereas sorghum and premix gave the lowest total mold counts (5481.25 and 2489.28, respectively) (Table 3.1).

The average total mold count for each sample of mixed poultry feeds and component raw materials in warm season was lower than that in the cold season (Table 3.3).

B. Total mold count at 25 °C and 35 °C

Total mold counts for all samples of mixed poultry feeds and component raw materials at 25 °C (Table 3.4) were higher than those at 35 °C (Table 3.5).

C. Frequency and abundance of external mycobiota

Frequency (% of positive samples) and abundance or level of contamination (CFU g^{-1} Dwt.) of the genera and species of fungi identified as external mycobiota of the 100 sample studied are presented in Table 3.6. These genera were represented by a large number of species and many of them were identified. Fifteen species and 5 genera were isolated.

Aspergillus

The most frequent mold, encountered in more than 50 % of the samples was *Aspergillus* which are the most common genus, emerged in 95 % of the samples contributing (74.1 %) of total fungi. Eleven species of *Aspergillus* were identified.

Among *Aspergillus* species, *A. restrictus* (93 %) was the most frequent, followed by *A. glaucus* (78 %), *A. candidus* (56 %), *A. flavus* (39 %) and *A. amstelodami* (36 %). These compromised 31.1 %, 15.5 %, 14 %, 1.25 % and 3 % of total fungi, respectively and 42 %, 21 %, 18.9 %, 1.7 % and 4 % of total *Aspergillus* isolates, respectively (Table 3.6).

A. terreus, A. niger, A. ochraceus, A. wentii and *A. aluteus* were less frequent and abundant encountered in 25 %, 16 %, 15 %, 14 % and 10 % of the samples, respectively, and compromising 6.5 %, 1.4 %, 0.89 %, 0.73 % and 0.19 % of total *Aspergillus*, respectively, and 4.82 %, 1 %, 0.66 %, 0.54 % and 0.12 % of total fungi, respectively (Table 3.6).

Fusarium

Fusarium was the second most frequent genus being found in 68% of the samples, and compromised 8.3 % of the total fungi present (Table 3.6).

Penicillium

Penicillium species ranked third in number of cases of isolation and was represented in nearly 55% of the samples, and compromised 7.1 % of the total fungi present (Table 3.6).

Mucor

Mucor was the fourth common genus. It was encountered in 53% of the samples, and compromised 7.4 % of the total fungi present (Table 3.6).

Rhizopus

Rhizopus was isolated in low frequency and appeared in 39% of the samples, and compromised 3.7 % of total fungi present (Table 3.6).

3.3.2 Internal mycobiota of yellow corn

For yellow corn kernels, the average total mold counts for internal mycobiota were (CFU per kernel) 4.1, and 2.1 and 2 in cold and warm periods, respectively (Table 3.3). Among *Aspergillus species, A. flavus* was the most frequent occurring in 100 % of the samples, and in 0.31 CFU per kernel.

The most frequent molds infecting yellow corn kernels were *A. flavus, A. niger, Fusarium* species, *A. glaucus, A. fumigatus* and

Rhizopus species, occurring in 100 %, 96 %, 92 %, 92 %, 92 and 90 %, of the kernels tested, respectively (Table 3.4).

3.4 *Aspergillus flavus* group

The average total count (CFU g^{-1} Dwt.) of *A. flavus* in all 100 samples was 2006.5, and compromised 1.25 % of total fungi and 1.7 % of total *Aspergillus* isolates (Table 3.6).

3.4.1 Aflatoxigenic *A. flavus* isolates (external mycobiota)

A. On CAM

Out of 100 samples analyzed, 114 *A. flavus* isolates screened, 62 (52.5 %) isolates showed fluorescence on CAM (Table 3.7).

B. On TLC

TLC analysis of the aflatoxins of different fungal isolates showed that, among 35 samples tested, and of the 114 *A. flavus* isolates screened, only 4 samples (11.4%) (Table 3.7) contained aflatoxins ($\leqslant 5$ ng g^{-1}) (Table 3.8).

Twenty-five aflatoxigenic isolates of *A. flavus* were found in yellow corn (external) and mixed poultry feeds. Sixteen of these isolates were screened for aflatoxins, only (12), and (9) isolates recovered from yellow corn and mixed poultry feeds samples, respectively showed fluorescence on CAM. None of the two isolates of *A. flavus* recovered from premix showed fluorescence on CAM, and none of the isolates of *A. flavus* recovered from soybeans and sorghum were aflatoxigenic.

The analysis of aflatoxins in yellow corn and mixed poultry feeds samples showed that aflatoxins B_2, and G_2 were the most predominant toxins detected.

3.4.2. Aflatoxigenic *A. flavus* isolates in yellow corn (internal mycobiota)

An average number (0.31 CFU kernel $^{-1}$) of *A. flavus* isolates was found in yellow corn (internal) of which (0.495 CFU kernel $^{-1}$) showed fluorescence on CAM. None of them were shown to be aflatoxigenic.

Table 3.1 Moisture content, total aerobic bacteria (CFU/gDwt.), abundance (external and internal) and incidence (% positive samples) on M2 and M5 media at 25 C and 35 C of mold species and genera in mixed poultry feeds and component raw materials

	External mycobiota (CFU/g Dwt.)										Internal mycobiota (CFU/kernel)	
	Mixed poultry feeds		Soybeans		Sorghum		Premix		Yellow corn		Yellow corn	
Number of samples	28		25		8		14		25		25	
Moisture content (%)	10.79		11.93		11.5756		6.79		11.69			
Total aerobic bacteria (CFU/g Dwt.)	93000		630000		235000		294000		311000			
Total mold count (CFU/g Dwt.)	30696.43		47640		5481.25		2489.285		73540		4.0520 CFU/ kernel	
External mycobiota	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence	Abundance
Aserqillus amstelodami	17	0.375	56	2.26	25	0.001	7	0.011	16	2.16	20	0.01
Aspergillus aluteus	0	0	4	0.06	0	0	57	0.143	4	0.02	20	0.03
Aspergillus candidus	64	5.16	80	9.42	63	0.9	7	0.007	44	6.88	76	0.066
Aspergillus flavus	36	0.286	44	0.68	25	0.009	14	0.007	56	0.94	100	0.31
Aspergillus fumigatus	14	0.089	16	0.48	0	0	14	0.039	48	2.8	92	0.284
Aspergillus glaucus	68	3.982	88	9.62	100	0.05	43	0.232	92	10.42	92	0.616
Aspergillus niger	39	0.964	8	0.14	25	0.009	0	0	8	0.26	96	1.046
Aspergillus ochraceus	7	0.072	36	0.64	0	0	0	0	12	0.34	64	0.194
Aspergillus restrictus	92	10.55	96	15.1	100	4.5	71	1.382	96	20.56	60	0.036
Aspergillus terreus	36	3.036	24	0.78	63	0.001	14	0.029	44	3.58	20	0.032
Aspergillus wentii	36	0.036	20	0.48	25	0.003	14	0.014	20	0.3	4	0.008
Fusarium species	43	1.875	68	3.14	38	0.006	36	0.332	52	7.88	92	0.96
Mucor species	71	3.305	80	2.14	88	0	50	0.047	76	4.14	84	0.106
Penicillium species	46	0.589	20	2.52	62	0.001	7	0.004	64	8.12	56	0.104
Rhizopus species	29	0.375	20	0.18	0	0	50	0.242	80	5.14	90	0.25
Total		**30.7**		**47.64**		**5.481**		**2.489**		**73.54**		**4.052**

Moisture content of any stored products should not exceed 12 %

Total mold count should not exceed the value of 10^6 CFU/g

Total aerobic bacteria should be at the intervals $10^{4.1}$-$10^{6.1}$ in premix and $10^{5.1}$-$10^{6.1}$ for other raw materials

Table 3.2 Abundance of external and internal mycobiota in mixed poultry feeds and components raw materials on M2 and M5 media at 35 ˚C and 25 ˚C

	External mycobiota (CFU/g Dwt.)																				Internal Mycobiota(CFU/ 10000 kernel)			
	Mixed poultry feeds				Soybeans				Sorghum				Premix				Yelow corn				Yelow corn			
Number of samples	28				25				8				14				25				25			
Total mold count (CFU/g Dwt.)	30696.428				47640				5481.25				2489.285				73540				40520			
Temperature (˚C)	35		25		35		25		35		25		35		25		35		25		35		25	
External mycobiota	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5	M2	M5
Asergillus amestelodami	17.857	35.714	0	321.43	300	480	780	700	0	0	0.625	0.625	0	10.714	0	0	600	1180	380	0	0	40	20	40
Aspergillus aluteus	0	0	0	0	0	0	60	0	0	0	0	0	7.1428	32.143	50	53.571	0	0	20	0	160	80	40	20
Aspergillus candidus	0	410.71	1053.6	3696.4	900	380	5780	2360	0.625	10.625	46.875	36.25	0	0	3.5714	3.5714	0	140	2580	4160	140	80	240	200
Aspergillus flavus	0	107.14	0	178.57	20	140	140	380	3.125	1.875	0	4.375	0	3.5714	0	3.5714	380	140	0	420	640	880	600	980
Aspergillus fumigatus	17.857	0	53.571	17.857	80	180	60	160	0	0	0	0	0	0	0	39.285	660	620	820	700	880	720	660	580
Aspergillus glaucus	1214.3	500	767.85	1500	4060	2180	1860	1520	5.625	18.125	9.375	16.875	7.1428	35.714	135.71	53.571	880	2040	3260	4240	1500	1620	1440	1600
Aspergillus niger	250	17.857	125	571.42	120	20	0	0	0.625	5	3.125	0	0	0	0	0	0	100	0	160	2660	2360	2400	3040
Aspergillus ochraceus	0	0	0	71.428	200	160	60	220	0	0	0	0	0	0	0	0	60	100	140	40	300	440	680	520
Aspergillus restrictus	750	1482.1	3875	4446.4	1600	1560	6100	5840	56.88	112.5	21.875	23.125	7.1428	0	553.57	821.42	920	2840	6240	10560	60	80	120	100
Aspergillus terreus	107.14	71.428	1071.4	1785.7	80	60	560	80	3.125	1.875	12.5	11.25	10.714	14.286	0	3.5714	1300	260	860	1160	20	20	120	160
Aspergillus wentii	0	0	35.714	0	360	0	60	60	0	0	0	3.125	3.5714	0	3.5714	7.1428	0	120	20	160	0	0	0	80
Fusarium species	0	107.14	785.71	982.14	20	140	1560	1420	0	0	1.875	4.375	0	0	92.856	239.28	40	180	1180	6480	2700	2120	2600	2180
Mucor species	1339.3	339.28	732.14	892.85	740	480	440	480	29.38	3.75	56.875	28.125	10.714	25	3.5714	7.1428	2160	1140	360	480	220	420	160	260
Penicillium species	35.714	53.571	357.14	142.86	20	20	1940	540	0	5.625	1.875	6.25	0	0	0	3.5714	180	20	2240	5680	120	220	400	300
Rhizopus species	0	0	125	250	20	20	60	80	0	0	0	0	3.5714	157.14	57.142	25	20	100	2360	2660	680	580	840	400
Total	3732.1	3125	8982.1	14857	8520	5820	19460	13840	99.38	159.38	155	134.38	50	278.57	899.99	1260.7	7200	8980	20460	36900	10080	9660	10320	10460

Table 3.3 Abundance of external and internal mycobiota in mixed poultry feeds and component raw materials in cold and warm seasons*

	External mycobiota (CFU/ g Dwt.)										Internal mycobiota (CFU/ kernel)	
	Mixed poultry feeds		Soybeans		Sorghum		Premix		Yellow corn		Yellow corn	
Number of samples	15	13	13	12	4	4	6	8	13	12	13	12
External mycobiota	Cold	Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold	Warm
Asergillus amestelodami	339.3	35.71	400	1860	12.5	0	10.71	0	2140	20	0.004	0.006
Aspergillus aluteus	0	0	0	60	0	0	128.6	14.29	0	20	0.028	0.002
Aspergillus candidus	3482	1679	6580	2840	150	793.8	0	7.143	6560	320	0.032	0.034
Aspergillus flavus	142.9	142.9	400	280	68.75	25	7.143	0	240	700	0.172	0.138
Aspergillus fumigatus	0	89.29	300	180	0	0	3.571	35.71	2340	260	0.154	0.13
Aspergillus glaucus	2429	1554	4460	5160	393.8	106.3	189.3	42.86	4660	5760	0.302	0.314
Aspergillus niger	482.1	660.7	40	100	12.5	75	0	0	280	0	0.534	0.512
Aspergillus ochraceus	53.57	17.86	420	220	0	0	0	0	200	140	0.082	0.112
Aspergillus restrictus	4554	6000	7540	7560	1638	506.3	1286	96.43	14420	6140	0.022	0.014
Aspergillus terreus	2875	160.7	60	720	200	87.5	0	28.57	3160	420	0.01	0.022
Aspergillus wentii	35.71	0	420	60	31.25	0	10.71	3.571	120	180	0	0.008
Fusarium species	214.3	1661	1380	1760	62.5	0	332.1	0	7800	80	0.484	0.476
Mucor species	1500	1804	660	1480	725	456.3	35.71	10.71	2720	1420	0.046	0.06
Penicillium species	428.6	160.7	2440	80	125	12.5	0	3.571	2960	5160	0.088	0.016
Rhizopus species	107.1	267.9	20	160	0	0	28.57	214.3	3520	1620	0.128	0.122
Total	16643	14232	25120	22520	3419	2063	2032	457.1	51120	22240	2.086	1.966

*Cold season: 1 October - 31 Mach. Warm season: 1 April - 30 September

Table 3.4 Abundance (external and internal) and incidence (% of positive samples) of mold species and genera in mixed poultry feeds and component raw materials on M2 and M5 media at 25 °C

	External mycobiota (CFU/g Dwt.)										Internal mycobiota (CFU/kernel)	
	Mixed feeds		Soybeans		Sorghum		Premix		Yellow corn		Yellow corn	
Number of samples	28		25		8		14		25		25	
Total mold count (CFU/g Dwt.)	23657.8		33300		2893.75		2153		57360		2.078	
External mycobiota	Incidence %	Abundance 1000	Incidence %	Abundance 1000	Incidence %	Abundance 1000	Incidence %	Abundance 1000	Incidence %	Abundance 1000	Incidence %	Abundance (CFU/kerne
Asergillus amestelodami	14.3	0.32	36	1.48	25	0.01	0	0	8	0.38	12	0.006
Aspergillus aluteus	0	0	4	0.06	0	0	50	0.1	4	0.02	12	0.006
Aspergillus candidus	53.6	4.75	72	8.14	50	0.83	7.1	0.01	44	6.74	52	0.044
Aspergillus flavus	17.9	0.18	40	0.52	25	0.04	7.1	0	32	0.42	44	0.158
Aspergillus fumigatus	10.7	0.07	8	0.22	0	0	14.3	0.04	40	1.52	76	0.124
Aspergillus glaucus	53.6	2.27	76	3.38	50	0.26	42.9	0.19	92	7.5	84	0.304
Aspergillus niger	32.1	0.7	0	0	12.5	0.03	0	0	4	0.16	100	0.544
Aspergillus ochraceus	7.1	0.07	32	0.28	0	0	0	0	12	0.18	60	0.12
Aspergillus restrictus	82.1	8.32	96	11.9	100	0.45	71.4	1.37	96	16.8	36	0.022
Aspergillus terreus	14.3	2.86	20	0.64	37.5	0.24	7.1	0	24	2.02	8	0.028
Aspergillus wentii	3.6	0.04	16	0.12	25	0.03	7.1	0.01	16	0.18	4	0.008
Fusarium species	39.7	1.77	68	2.98	37.5	0.06	35.7	0.33	36	7.66	100	0.478
Mucor species	60.7	1.63	48	0.92	50	0.85	14.3	0.01	48	0.84	64	0.042
Penicillium species	28.6	0.5	16	2.48	25	0.08	7.1	0	64	7.92	48	0.07
Rhizopus species	28.6	0.38	16	0.14	0	0	50	0.08	76	5.02	72	0.124
Total		23.8		33.3		2.89		2.15		57.4		2.078

Table 3.5 Abundance (external and internal) and incidence (% of positive samples) of mold species and genera in mixed poultry feeds and component raw materials on M2 and M5 mediaat 35 °C

	External mycobiota (CFU/g Dwt.)										Internal mycobiota (CFU/kernel)	
	Mixed feeds		Soybeans		Sorghum		Premix		Yellow corn		Yellow corn	
Number of samples	28		25		8		14		25		25	
Total mold count (CFU/g Dwt.)	6857		14340		2587.35		328.27		1618		1.974	
Mycobiota	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence %	Abundance 1000g	Incidence %	Abundance (CFU/kernel
Asergillus amestelodami	7.1	0.05	32	0.78	0	0	7.1	0.01	12	1.78	8	0
Aspergillus aluteus	0	0	0	0	0	0	28.5	0.04	0	0	12	0.02
Aspergillus candidus	21.4	0.41	28	1.28	50	0.11	0	0	8	0.14	32	0.02
Aspergillus flavus	17.9	0.16	12	0.16	25	0.05	7.1	0	36	0.52	48	0.15
Aspergillus fumigatus	3.6	0.02	12	0.26	0	0	0	0	20	1.28	88	0.16
Aspergillus glaucus	39.3	1.76	80	6.24	75	0.24	14.3	0.04	68	2.92	80	0.31
Aspergillus niger	21.4	0.25	8	0.14	25	0.06	0	0	4	0.1	100	0.5
Aspergillus ochraceus	0	0	16	0.36	0	0	0	0	8	0.16	44	0.07
Aspergillus restrictus	67.9	2.2	68	3.16	87.5	1.69	14.3	0.01	68	3.76	20	0.01
Aspergillus terreus	17.9	0.17	20	0.14	50	0.05	7.1	0.03	24	1.56	8	0
Aspergillus wentii	0	0	8	0.36	0	0	7.1	0	1	0.12	0	0
Fusarium species	7.1	0.16	12	0.16	0	0	0	0	8	0.22	100	0.48
Mucor species	53.6	1.6	68	1.22	75	0.33	42.9	0.04	64	3.3	76	0.06
Penicillium species	14.3	0.08	4	0.04	37.5	0.06	0	0	8	0.2	28	0.03
Rhizopus species	0	0	8	0.04	0	0	7.1	0.16	12	0.12	68	0.13
Total		6.86		14.3		2.59		0.33		16.2		1.97

Table 3.6 Abundance of external mycobiota (CFU/g Dwt.) in cold and warm seasons* and incidence (% of positive samples)of 100 samples of mixed poultry feeds and component raw materials

External mycobiota	Cold	Warm	Abundance	Incidence	% of total fungi	% of total *Aspergillus*
Asergillus amstelodami	2643.6	1915.7	4818.21	36	3	4
Aspergillus aluteus	2902.5	94.286	222.856	10	0.12	0.19
Aspergillus candidus	29437	5639.5	22411.6	56	14	18.9
Aspergillus flavus	5953.6	1147.9	2006.61	39	1.25	1.7
Aspergillus fumigatus	814.64	565	3208.57	29	2	2.71
Aspergillus glaucus	858.75	12623	24754.3	78	15.5	21
Aspergillus niger	9788.9	835.71	1650.36	16	1	1.4
Aspergillus ochraceus	16772	377.86	1051.43	15	0.66	0.89
Aspergillus restrictus	673.57	20303	49739.4	93	31.1	42
Aspergillus terreus	5640.7	1416.8	7711.79	35	4.82	6.5
Aspergillus wentii	6295	243.57	861.25	14	0.54	0.73
Fusarium species	12132	3500.7	13289.6	68	8.3	
Mucor species	617.68	5170.5	10811.3	53	7.4	
Penicillium species	3675.7	5416.8	11370.4	55	7.1	
Rhizopus species	128.57	2262.1	5937.85	39	3.7	
Total	98334	61512	159845			

* Cold season: 1 October - 31 March. Warm season: 1 April - 30 September

Table 3.7 Occurrence of aflatoxigenic *A. flavus* isolates and aflatoxins in mixed poultry feeds and component raw materials.

	Poultry feeds	Soybeans	Sorghum	Premix	Yellow corn	Total
Number of *A. flavus* isolates screened/Number of samples examined	(16/28)	(34/25)	(15/8)	(2/14)	(47/25)	(114)
Number of *A. flavus* isolates showing fluorescence on CAM	9	16	2	0	35	62
Number of aflatoxins-positive samples	2	0	0	0	2	4

Table 3.8 Determination of aflatoxins level (ppb)

Aflatoxins standard	Diameter (mm) of 50 ng/ml	Samples	Diameter (mm) - (Aflatoxin)	Aflatoxins level (ppb)
G1	0.4	Mixed poultry feeds	0.34 - (G2)	1.7
G2	0.4	Mixed poultry feeds	0.85 - (B2)	50
B1	0.34	Yellow corn (external)	0.4 - (G2)	0.7
B2	0.34	Yellow corn (external)	0.12 - (B2)	2

CHAPTER FOUR
DISCUSSION

CHAPTER FOUR

DISCUSSION

This study was carried out in cold and warm seasons so as to determine the influence of moisture content and some environmental factors on the microbiological and bacteriological quality of mixed poultry feeds and component raw materials.

In this study the moisture content of mixed poultry feeds and component raw materials did not show any noteworthy differences, the average moisture content of all the samples was low and ranged between 6.8% - 11.9% during all the sampling period. This is an acceptable result and may play a major role in decreasing the contamination level of mixed poultry feeds and component raw materials.

The generally high total mold count and total aerobic count in soybeans and yellow corn may be partially due to a relatively high moisture content (11.9 %, and 11.7 %, respectively) and higher protein content. Also an elevated level of carbohydrate and/ or lipids is favourable for *A. flavus* and aflatoxins production (Bars & Bars, 1998). The relatively low total mold count in premix was probably due to low moisture content, which prevented growth and development of fungi (Bauduret, 1990). These results may indicate that there is a relationship

between moisture content, contamination, total mold count and total aerobic bacteria.

Contamination of animal feeds and component raw materials by fungi, which impair the quality of feedingstuffs and in certain conditions may produce aflatoxins. Aflatoxins that were found as a residue in animal feeds may endanger human health because of carcinogenic, teratogenic and immunosuppressive properties (Vengust *et al.*, 1998). They also have a significant impact on economics, by causing losses in farm animals, suppress growth and productivity, or giving rise to difficulties in their management, or by rendering commodities unacceptable in national and international trade because they do not conform with existing regulations (Pittet, 1998).

High total counts of mold and bacteria in soybeans and yellow corn may be attributed to several reasons: (1) Probably yellow corn is growing in climates where it is likely to have perennial contamination, and it is the stable food in many countries. (2) Pre-harvest contamination from soil; as food may be contaminated with any of the organisms present in the soil in which they are grown. (3) High densities in the field during production, processing, transport and storage. (4) The cleanliness of irrigation water (mold growth exceed critical value). (5) Post-harvest contamination which occur if crop drying is delayed. (6) Improper hygiene methods during storage and handling of crops. (7) Insect and

rodent infestations facilitate mold invasion of some stored commodities (Wyllie & Morchause, 1978).

The existence of contaminated animal feeds has negative effects on food consumption, and nutrition. Harmful effects of aflatoxins on human and animal health have been well recognized although some vital statistics on aflatoxins-induced diseases are generally lacking and much work is still needed to evaluate toxicological effects of aflatoxins (Boutrif & Canet, 1998).

Different weather conditions play a major role in determining the numbers of total mold count and total aerobic bacteria. For each sample of mixed poultry feeds and component raw materials in the present work, total mold count was found to be higher in cold season than in warm season (Table 3.3). This could be attributed to higher moisture content during the rainy cold season (Bauduret, 1990). But it is not possible to compare the current results with those obtained elsewhere by other authors, because of different weather conditions (Bauduret, 1990).

The total mold count in mixed poultry feeds had a relatively lower count than in the yellow corn and soybeans samples. This may be due to granulation of mixed poultry feeds, which improve their mycological quality (Bauduret, 1988). Granulation of contaminated component with uncontaminated one may reduce or even remove any light–weight, infected kernels and thus further reduce of aflatoxin concentrations, as

aflatoxins are associated with broken or light–weight kernels (Wyllie & Morchause, 1978). Raw materials should be separated from finished products to prevent bacterial and mold transfer. Blending or mixing damaged or lower quality crops in separate trunks or trailers.

High total aerobic bacteria and total mold count occurred in yellow corn and soybeans, but much lower count was found in the formulated mixed poultry feeds, presumably due to dilution (Dalcero *et al.*, 1997), and incorporation of other components in the formulated mixed poultry feeds (Wyllie & Morchause, 1978).

In the present work, all the samples of mixed poultry feeds and component raw materials were negative for *Salmonella*, but failure to demonstrate its presence in all samples does not indicate that such bacteria were not present. Absence of *Salmonella* in the consignment may still involve occurrence of other enteric pathogens (Drion & Mossel, 1997). However there were no obvious association between these aflatoxins content and bacteriological analysis (Candlish *et al.*, 1998).

However the majority of animals feeds and component raw materials analysis for aflatoxins did not contain any detectable level of this toxin. The highest levels of aflatoxins (3 samples out of 4) were found in yellow corn and mixed poultry feeds during cold season. This might be due to unsuitable storage conditions.

The levels of aflatoxins B_2 and G_2 in yellow corn and mixed poultry feeds which is a trace but detectable level ($< 5ng\ g^{-1}$) did not exceed the standard levels ($20\ ng\ g^{-1}$). Even if the amount of aflatoxins produced in this partial study is not considerably high enough to cause adverse effects in animals it is a sign that the feed will be lower nutritionally and may still lead to serious health problems to both animals and humans (Dalcero *et al.*, 1997).

No correlation was detected between external and internal mycobiota, and between abundance of *A. flavus* and aflatoxin levels in yellow corn. One does not know whether the fungi represented surface contamination of yellow corn kernel or were fragments and spores from inside the yellow corn kernel (Hesseltine *et al.*, 1995). Grains that are not moldy can be contaminated by aflatoxins, as fungi can grow in the inner part of the grain or the aflatoxins are on the grain even if the fungus is not visible or found there any more (Scussel *et al.*, 1998).

Total mold count and the abundance of *Aspergillus* species isolated from soybeans samples were relatively high. But no aflatoxins could be detected considering that there was no correlation between total mold count and the amount of aflatoxin (Bean *et al.*, 1972). A good correlation seemed to exist between the presence of at least one toxigenic strain and presence of aflatoxins in the sample (Table3.7) (Bauduret, 1990; Dalcero *et al.*, 1997).

This study indicates that yellow corn can be a suitable substrate for growth of aflatoxin-producing strains of *Aspergillus* species. So that routine examination of storage raw materials crops for the presence of aflatoxins, especially where raw materials will be used in poultry or livestock feeds should be done.

Total mold count enumerated on M5 media were higher than M2 media, this may be due to different or variant culture composition of both media (Bars & Bars, 1998). Total mold count for all the samples at 25 °C were higher than total mold count at 35 °C, except for premix. The optimum temperature for *A. flavus* growth is 35 °C but maximum aflatoxin biosynthesis occurs at 25 – 30 °C.

In the West Bank, where animal feeds lack adequate quality control checks and also where storage conditions favour fungal growth. This study showed that external mycobiota enumerated in mixed poultry feeds approached those observed in Argentina (Dalcero *et al.*, 1998), but with a higher incidence of, *A. restrictus* and *A. candidus* and with a lower total mold count and incidence of *Fusarium* species. Fungi identified in poultry feeds were very close to those observed in Spain (Romo & Fernandez, 1986) and in Reunion Island (Bauduret, 1990), but with a higher incidence of, *A. restrictus* and *Mucor* species and with a lower incidence of *A. flavus*, *A. niger*, *A. glaucus* and *Penicillium* species. On the other hand, the percentage of aflatoxigenic isolate in poultry feeds

was (11.4%) which is lower than those observed in Spanish poultry feeds (39%) (Romo & Fernandez, 1986) and in Reunion island (44%) (Bauduret, 1990).

Of the 114 *A. flavus* isolates screened in this survey, 4 (6.22%) were aflatoxigenic. Among 35 animal feed samples containing at least one fluoresce isolate on CAM, 4 contained aflatoxins. So, the CAM screening method appears to be a good microbiological examination routine to define risks of aflatoxin presence or absence in samples. Again, a good correlation seemed to exist between presence of at least one toxigenic isolate and presence of aflatoxins in the samples.

Fungi identified in yellow corn kernels (external) were similar to those determined in Argentina (Dalcero *et al.*, 1998), Spain (Romo & Fernandez, 1986) and Reunion Island (Bauduret, 1990). However a lower incidence and abundance of *Fusarim* species, *A. niger* and *A. flavus,* and a higher incidence of *A. restrictus*, *A. glaucus*, *Mucor* species, *Rhizopus* species and *Penicillium* species, was noticed in this work.

Mycobiota in soybeans of this study were similar to those observed in France (Bauduret, 1988), but with a higher incidence of *A. flavus, A. candidus*, *Mucor* species, *Fusarium* species and *Rhizopus* species, and with a lower incidence of *A. glaucus*, *A. niger*, *A. fumigatus* and *Penicillium* species.

Molds genera and species identified in premix were never abundant due to low moisture content.

No correlation seemed to occur between external and internal abundance of mycoflora in yellow corn and the presence of aflatoxigenic *A. flavus*.

Fungi identified in yellow corn were similar to those determined in France (Herry et al., 1987) and Reunion Island (Bauduret, 1990), but we noticed a higher incidence and abundance of *A. restrictus* group, and lower incidence and abundance of *Fusarium* species.

The results of this study suggest that high incidence of aflatoxigenic *A. flavus* fungi production in mixed poultry feeds and component raw materials if stored improbably could be indicating a potential mycotoxin problem in the West Bank poultry feeds. Further research may be needed in the future regarding risk assessment on aflatoxins.

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Appendix A

(Media, Stains & Reagents)

Coconut Agar Medium (CAM) (Davis *et al.*, 1987)

Agar	4.0 gm
Shredded coconut	100 gm
Distilled water	200 g

Shredded coconut was homogenate for 5 minutes in hot distilled water, then filtered through four layers of cheese cloth, and the pH of the filtered was adjusted to pH 7 with 2N NaOH.

M2 media (Bauduret, 1990)

Malt extract	2.0 %
Yeast extract	0.2 %
Agar	1.8 %
Chloramphenicol	60 mg/ml
Gentamycine sulfate	50 mg/ml

The antibiotics were added at temperature between 42 - 48 °C.

M5 media (Bauduret, 1990)

Malt extract	5.0 %
Yeast extract	0.2 %
Sodium chloride	5.0 %
Agar	1.8 %
Chloramphenicol	60 mg/ml
Gentamycine sulfate	50 mg/ml

Solution A (Bauduret, 1990; Herry *et al.*, 1987)

Tryptone	1.6 gm
Sodium chloride	8.5 gm
Tween 80	2 drops
Distilled water	1000 ml

Potato Dextrose Agar (Koneman & Roberts, 1985)

Potato infusion	200 gm
Dextrose	20 gm
Agar	15 gm
Distilled water	1000 ml

Sabouraud's Dextrose Agar (Koneman & Roberts, 1985)

Dextrose	20 gm
Peptone	10 gm
Distilled water	1000 ml
Agar	17 gm
Chloramphenicol	(0.005%)
Cycloheximide	(0.05%)

Lactophenol-Aniline Blue Stain (Koneman & Roberts, 1985)

Distilled water	20ml
Lactic acid	20 ml
Phenol crystals	20 gm
Aniline blue	0.05 gm
Glycerol	40 ml

Dissolve phenol in lactic acid, glycerol, and water by gently heating, then add aniline blue.

Plate-Count Agar (PCA) (Bauduret, 1990)

Tryptone	5.0 gm
Yeast extract	2.5 gm
Dextrose	1.0 gm
Agar	9.0 gm
Distilled water	1000 ml

Appendix B

Average moisture content (%)* and abundance (CFU/g Dwt.) of external mycobiota in mixed poultry feeds and component raw materials on M2and M5 media at 25 ˚C and 35 ˚C

Yellow corn					Premix					Soybeans					Mixed poultry feeds					Sorghum				
Dwt. of 2 replica sample(g)		average(g)	*	Abundance	Dwt. of 2 replica sample(g)		average(g)	*	Abundance	Dwt. of 2 replica sample(g)		average(g)	*	Abundance	Dwt. of 2 replica sample(g)		average(g)	*	Abundance	Dwt. of 2 replica sample(g)		average(g)	*	Abundance
17.67	17.66	17.665	11.675	4080	18.43	18.42	18.425	7.875	25	17.61	17.6	17.605	11.975	2240	17.86	17.88	17.87	10.65	2000	17.764	17.76	17.762	11.19	593.75
17.72	17.716	17.7205	11.3975	6460	18.42	18.422	18.421	7.895	0	17.6	17.61	17.605	11.975	2960	17.9	17.89	17.895	10.525	410.714	17.764	17.69	17.727	11.365	425
17.67	17.66	17.665	11.675	860	18.5	18.52	18.51	7.45	0	17.6	17.6	17.6	12	400	17.9	17.91	17.905	10.475	357.14	17.76	17.79	17.77	11.15	300
17.671	17.67	17.67	11.65	2860	18.4	18.34	18.37	8.15	310.7118	17.6	17.56	17.59	12.05	5960	17.9	17.87	17.885	10.575	1642.857	17.6	17.18	17.38	13.1	337.5
17.67	17.7	17.685	11.575	1000	18.41	19.32	19.365	3.175	57.1424	17.61	17.857	17.59	12.05	680	17.86	17.776	17.818	10.91	232.142	17.66	17.56	17.61	11.95	1406.25
17.66	17.76	17.665	11.675	1840	18.56	18.9	18.73	6.35	64.2852	17.66	17.7	17.68	11.6	3720	17.9	17.91	17.905	10.475	35.714	17.7	17.7	17.7	11.5	125
17.6	17.66	17.63	11.85	1000	18.93	19	18.965	5.175	0	17.66	17.73	17.695	11.525	280	17.9	17.83	17.87	10.65	53.571	17.76	17.77	17.765	11.175	1756.25
17.6	17.6	17.6	12	2800	18.422	18.5	18.461	7.695	350	17.6	17.56	17.58	12.1	1500	17.9	17.88	17.89	10.55	250	17.77	17.76	17.765	11.175	537.5
17.54	17.5	17.52	12.4	4520	18.5	18.41	18.455	7.725	0	17.57	17.54	17.555	12.225	1200	17.89	17.9	17.9	10.5	17.857	17.72225	17.64875	17.68488	11.575625	5481.25
17.6	17.6	17.6	12	8520	15.9	18.83	18.865	5.675	385.7112	17.66	17.51	17.585	12.075	680	17.89	17.79	17.84	10.8	1732.142					
17.3	17.49	17.395	13.025	4860	18.483	18.48	18.481	7.595	207.1412	17.51	17.3	17.405	12.975	3220	17.98	17.93	17.96	10.2	2785.714					
17.89	17.9	17.895	10.525	5800	18.59	18.6	18.595	7.025	157.1416	17.9	17.7	17.8	11	1440	17.77	17.7	17.735	11.325	2035.714					
17.682	17.74	17.711	11.445	6480	19.9	18.68	18.79	6.05	407.1396	17.63	17.5	17.57	12.15	1360	17.4	17.74	17.57	12.15	375					
17.77	18.1	17.935	10.325	560	18.55	18.54	18.545	7.275	525	17.63	17.62	17.625	11.875	1420	17.7	17.34	17.52	12.4	3482.142					
17.67	17.7	17.685	11.575	1320	18.428214	18.640143	18.641286	6.7935714	2,489.27	17.6	17.6	17.6	12	160	17.8	17.76	17.78	11.1	339.285					
17.67	17.668	17.669	11.655	2000						17.6	17.65	17.625	11.875	1560	17.89	17.81	17.85	10.75	982.142					
17.668	17.7	17.684	11.58	620						17.66	17.6	17.683	11.585	2420	17.89	17.91	17.9	10.5	125					
17.66	17.68	17.67	11.65	1280						17.54	17.31	17.425	12.875	1360	17.9	17.91	17.905	10.475	3642.857					
17.5	17.3	17.4	13	3280						17.8	17.77	17.785	11.075	2720	17.88	17.91	17.895	10.525	107.142					
17.74	17.77	17.755	11.225	1960						17.6	17.61	17.605	11.975	1100	17.8	17.59	17.695	11.525	767.857					
17.668	17.68	17.674	11.63	1440						17.61	17.66	17.635	11.825	1920	17.88	17.97	17.925	10.375	1553.571					
17.68	17.67	17.675	11.625	1880						17.6	17.63	17.615	11.925	3880	17.93	17.91	17.92	10.4	589.285					
17.66	17.67	17.665	11.675	1980						17.86	17.84	17.85	10.75	1320	17.9	17.61	17.755	11.225	910.714					
17.6	17.61	17.605	11.975	3420						17.4	17.54	17.425	12.875	2640	17.93	17.8	17.865	10.675	517.857					
17.74	17.7	17.72	11.4	2720						17.63	17.6	17.615	11.925	1500	17.88	17.79	17.835	10.825	607.142					
17.65196	17.67616	17.66234	11.6883	73540						17.6296	17.60788	17.61392	11.9304	47640	18	17.89	17.945	10.275	428.568					
															17.88	17.88	17.88	10.6	1875					
															17.9	17.81	17.855	10.725	2839.285					
															17.864643	17.817714	17.841714	10.79143	30696.412					

بسم الله الرحمن الرحيم

دراسة ميكروبيولوجيه لأعلاف الدواجن مع التركيز على فطر اسبرجلس فلافس المنتج للأفلاتوكسين.

رنا راضي كامل البرق

إشراف

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الملخص

تم جمع مائة عينة من اعلاف الدواجن و مكوناتها من المواد الخام (٢٨ عينة من الخلطة الجاهزة من العلف، ٢٥ فول صويا، ٢٥ ذرة صفراء، ٨ ذرة سورحم و ١٤ من الوحبة الكاملة من الفيتامينات المعززة). جمعت العينات عشوائياً من ٨ مصانع للأعلاف في منطقة الضفة الغربية خلال فترة ١٢ شهر (تموز ١٩٩٧- حزيران ١٩٩٨). تم فحص العينات و دراستها لمعرفة العدد الكلي للبكتيريا و الفطر و انواعها مع التركيز على بكتيريا السالمونيلا و فطر الاسبرجلس فلافس و ذلك بوساطة عدة طرق مرجعية للاكثار و الزراعة و الاختبارات البيوكيميائية و السيرولوجية. لقد اظهرت الدراسة خلو جميع العينات المفحوصة من السالمونيلا. كما اظهرت النتائج ان عينات الذرة الصفراء و فول الصويا قد اشتملتا على العدد الاكبر من البكتيريا ٦,٣ °، ٣,١١ ° وحدة تكاثر لكل غرام وزن جاف، على التوالي و ايضاً العدد الكبر من الفطر ٤٧٦٤٠، ٧٣٥٤٠ وحدة تكاثر لكل غرام وزن جاف، على التوالي. وقد عزى هذا الى ارتفاع مستوى الرطوبة فيهما نسبياً وارتفاع نسبة الكربوهيدرات والبروتينات والدهنيات فيهما ايضاً. اما بالنسبة للفيتامينات المعززة فقد اشتملت على العدد الاقل من الفطر وعزى هذا الى انخفاض مستوى الرطوبة فيها. واظهرت النتائج ان العدد الكلي للبكتيريا والفطر كان اعلى في الفصل البارد منة في الفصل الدافئ وعزى هذا الى ارتفاع نسبة الرطوبة في الفصل البارد الماطر. وتم في هذة الدراسة عزل ١٥ نوعا من الفطر تنتمي الى خمسة اجناس من العينات تحت الدراسة. وكان الفطران السائدان من هذة الفطريات ينتميان لفطري الاسبرجلس و الفيوزاريوم حيث كانا يشكلان ٩٥ % و ٦٨ % من جميع الفطريات المعزولة، على التوالي. من صنف الاسبرجلس عُرِّفَ ١١ صنف منها *Aspergillus restrictus* ، *A. glaucus* ، *A.candidus* ، *A. flavus*. وبلغ عدد عزلات اسبرجلس فلافس المعزولة ١١٤ عَزْلة (من ٣٥ عينه علف). وقد اظهر الفحص (اختبار Fluorescence للعزلات النامية على آجار حوز الهند CAM) ان ٦٤عزلة من هذة العزلات منتجة للأفلاتوكسين . وظهر من دراسة العينات (ال ٣٥) التي عُزل منها الاسبرجلس المنتج للأفلاتوكسين، بواسطة جهاز كروماتغرافيا الشرائح الرقيقة، ان ٤ عينات (٢ من الخلطة الجاهزة، ٢ من الذرة الصفراء) كانت ملوثه بالافلاتوكسين من نوع B ٢ , G٢ والتي تراكيزها (≤ من ٥ نانو غرام / غرام). مع العلم ان هذا التركيز اقل من مستوى المعايير والمواصفات العالمية (<٢٠ نانو غرام / غرام).