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Occurrence of Aflatoxigenic Fungi and Levels of Aflatoxin B1 in Foodstuff Marketed in Palestine

وجود الفطريات المفرزة للأفلاتوكسينات ومستويات أفلاتوكسين B1 في عينات غذائية من السوق الفلسطينية

This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree in "*Environmental Biology"* from the Faculty of Graduate Studies at Birzeit University, Palestine.

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Declaration

This is my original work and has never been submitted in part or whole for an award in any institution.

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Dedication

The hard work of this thesis is dedicated to my Mother for her countless moral contributions and physical support during the entire duration of the program.

To my father who always wanted to see me excel further and whose tear of happiness would strike like a bullet.

To my Husband who bared with me these three hard years.

To my daughter Salma whom constantly asked “Are you done yet mom?”

Last but not least to my family and friends

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List Of Abbreviations

ADBM: *Aspergillus* Differentiation Base Medium

AF: Aflatoxin

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

AFM1: Aflatoxin M1

APA: Aflatoxin-Producing Agar

AV: Ammonia Vapor

CAM: Coconut Agar medium

CFUs: Colony Forming Units

DNA: Deoxyribonucleic Acid

ELISA: Enzyme Linked Immunosorbent Assay

FAIRS: Food and Agricultural Import Regulations and Standards

FB: Fluorescence Based

GC: Gas Chromatography

LC: Liquid Chromatography

HACCP: Hazard Analysis and Critical Control Point

HCC: Hepatocellular Carcinoma

HPLC: High Performance Liquid Chromatography

IAC: Immuno Affinity Column

IARC: International Agency for Research on Cancer

OD: Optical Density

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

ppb: Parts per Billion

RIA: Radioimmunoassay

R-PDA: Rose Bengal Potato Dextrose Agar

RT-PCR: Real Time Polymerase Chain Reaction

SNP: Single Nucleotide Polymorphism

TLC: Thin Layer Chromatography

USFDA: U S Food and Drug Administration

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1. Summary

The basic Palestinian cuisine consists mainly of cereals (wheat, rice and corn) and other cereal-based foods, nuts and spices. Aflatoxigenic fungi are ubiquitous and tend to thrive in food products under favorable conditions. As their name implies, aflatoxigenic fungi produce aflatoxins that cause hepatic cancer threatening human and animal welfare. Therefore, the aim of this study is to evaluate the occurrence of aflatoxigenic fungi and the levels of aflatoxins, specifically Aflatoxin B1 (AFB1), in foods. It is of great importance to human and animal health as food and feed are prone to contamination. Besides, monitoring aflatoxins in foodstuffs enables government proper implementation of management policies and strategies that reduce the risks of contamination during storage, the economic losses due to contamination and the health care expenses associated with affected people.

In the present study, 229 food samples, belonging to 8 different food categories, marketed in the West Bank, Palestine were randomly collected from different retailers and were analyzed for the occurrence of fungi, with specific focus on *Aspergillus* spp. and for the levels of Aflatoxin B1. Samples analyzed were belonging to the following food categories: spices (118), grains and cereals (25), nuts and seeds (23), dried fruit (14), coffee (10), herbs/tea (9), tobacco and mua'ssel (15), and others (15) which consist of corn oil samples (6), peanut butters (3), chocolate spreads (3) and cocoa powder (3).

Total fungal contamination of food samples was done using Rose Bengal Potato Dextrose Agar "R-PDA". Of the analyzed samples (163), 96.3% were found contaminated with fungus

with a mean count of 1.0 ±0.4 $x10^5$ CFUs/g. The two food samples with the highest number of mold colonies recorded were chili ($2.5x10^6$ CFUs/g) and tobacco ($2.0x10^6$ CFUs/g) in addition to a sample of dried fruit with a high yeast count of ($5.9\ x10^6$ CFUs/g). Fungi belonging to 8 different genera were identified from all food samples analyzed. *Aspergillus* spp were the most frequently occurring among the species recording 69.3%. *Cladosporium* spp., *Penicilium* spp., Yeast, *Rhizopus* spp. and *Mucor* spp. made up 35.0, 23.3, 16.6, 16.6 and 6.7%, respectively. Of the species identified, 3.7% were from the rare occurring genera of *Alternaria* and *Trichoderm*. In addition, 31.3% of the occurring species were unidentifiable and categorized as unknown. By category, grains and cereals were found to be frequently contaminated with *Aspergillus* species where 92% of the samples were found infested. In decreasing order 84% of spices, 78% of tobacco, 71% of dried fruit, 56% of tea and herbs, 50% of coffee, 48% of nuts and seeds, and 8% of other uncategorized samples were contaminated with *Aspergillus* species. Of the genus *Aspergillus*, *A. niger* followed by *A. flavus* were dominating species occurring in 33% and 20% of the samples, respectively. The green *Aspergillus* spp. isolates were found mainly in chicken spices (19%) and red chili powder (16%). The presence of *A. fumigates*, a clinically important species that produces gliotoxin, was recorded in some commodities of sumac, pepper, ginger and mixed herbs.

Three different assessments were used to test aflatoxigenic ability of the green *Aspergillus.* These included the *Aspergillus* Differentiation Base Medium "ADBM" and the Coconut Agar Medium (CAM) where the colonies cultured in the latter were exposed to fluorescence and to ammonia vapor. Of the 22 tested isolates, 20 (90.9%) were identified to produce aflatoxin by both ADBM and CAM using the ammonia vapor method. On the other

hand, thirteen strains (59.1%) were found to produce aflatoxins by the fluorescence-based method on CAM.

Of the food samples, one hundred and seventeen samples were screened for AFB1 by enzyme-linked immunosorbent assay (ELISA). Occurrence of AFB1 was detected in 46 food samples (38.8%) out of 116 food commodities assessed. Levels of AFB1 ranged between 0.40 to 13.5 µg/kg with a mean value of 4.62±3.54 µg/kg with a 95% CI of 1.10 µg/kg. Maximum AFB1 levels were detected in red chili powder ranging from 4.15 to 13.5 µg/kg. Of the assayed food products, which were positive for AFB1, 49% (22 samples) were found to have levels above the permissible limits of 5µg/kg.

Water activity (a_w) of the food samples analyzed did not exceed the average of 0.522±0.051. In addition, no significant correlation was found between AFB1 levels, colony counts and water activity in the analyzed samples.

In conclusion the results of this study provide further evidence on the necessity to monitor imported as well as local food regularly for the presence of aflatoxin.

ملخص

تشكل الحبوب، كالقمح والأرز والذرة، ومشتقاتها بالإضافة للتوابل والبهارات والمكسرات، جزءا أساسيا من مكونات الطبق الفلسطيني. الفطريات المفرزة لسموم الأفلاتوكسين تنتشر عالميا ولها القدرة على النمو والتكاثر بكثرة على المواد الغذائية إذا ما توفرت الظروف البيئية المناسبة. سموم الأفلاتوكسين المفرزة من قبل هذه الفطريات تسبب سرطان الكبد وبالتالي تهدد حياة الإنسان والحيوان. لذلك، فإن الدراسات التي تعنى بمراقبة الفطريات والأفلاتوكسينات (وخاصة أفلاتوكسين بي 1) الملوثة للمواد الغذائية البشرية والحيوانية تعتبر غاية في الأهمية من أجل صحة الإنسان والحيوان. بالإضافة لذلك، فإن الدراسات هذه تمكن الحكومات من اتخاذ القرارات وتبني السياسات والاستراتيجيات للحد من تلوث المواد الغذائية بالسموم الفطرية وبالتالي الحد من الخسائر الاقتصادية التي تسببها هذه السموم نتيجة تلويثها للغذاء وبسبب الأضرار الصحية التي تسببها لأفراد المجتمع المتضررين من هذه السموم.

في هذه الدراسة، تم جمع 229 عينة عشوائية من مواد غذائية منتمية لثماني مجموعات غذائية مطروحة للبيع في الأسواق الفلسطينية وذلك من أجل فحص وجود الفطريات فيها وخاصة السامة مثل فطر (*Aspergillus*) ومن أجل تحليل مستويات الأفلاتوكسين بي 1 فيها. العينات الغذائية التي تم جمعها تنتمي للمجموعات التالية: الحبوب (118)، المكسرات (25)، الفواكه المجففة (14)، القهوة (10)، الشاي والأعشاب الطبية (9)، التبغ والمعسل (15)، عينات أخرى مثل زيت الذرة، زبدة الفستق، شوكولاتة دهن، شوكولاتة بودرة (15).

تم فحص وجود الفطريات في عينات الغذاء باستخدام وسائط زراعية مختلفة، حيث تبين أن 96.3% من العينات ال 163 التي تم فحصها ملوثة بالفطريات وبمعدل يساوي $1.0 \pm 0.4 \text{ X } 10^5$ خلية فطرية لكل غرام. العينات الغذائية الأكثر تلوثا بالفطريات كانت الفلفل الأحمر الحار ($2.5 \text{ X } 10^6$ خلية فطرية لكل غرام) يليه التبغ ($2.0 \text{ X } 10^6$ خلية فطرية لكل غرام). بالإضافة لذلك، فقد تبين أن الفواكه المجففة ملوثة بالخمائر بمعدل يصل إلى $5.9 \text{ X } 10^6$ خلية خمائر لكل غرام.

أما الفطريات التي تم عزلها، فقد تبين أنها تنتمي ل 8 أجناس من الفطريات. حيث تبين أيضا أن فطر (*Aspergillus*) هو الأكثر شيوعا من بينها حيث سجل وجوده في 69.3% من العينات. أما الأجناس الأخرى فقد وجدت بمعدلات أقل كما يلي: *Cladosporium* (35%)، *Penicillium* (23.3%)، Yeast (16.6%) *Rhizopus*، *Mucor* (6.7%)، أجناس أخرى نادرة (*Alternaria* and *Trichoderm*)، 3.7%. هذا بالإضافة ل 31.3 % لم يتم التعرف عليها.

كذلك، بينت الدراسة أن 92% عينات الحبوب ملوثة بفطريات ال (*Aspergillus*) يليها عينات البهارات (84%)، التبغ والمعسل (78%)، الفواكه المجففة (71%)، الشاي والأعشاب الطبية (56%)، القهوة (50%)، المكسرات (48%) اما العينات الأخرى فكانت ملوثة بنسبة 8%.

أما أنواع الفطريات من جنس (*Aspergillus*) فقد تبين أن الأنواع *A. niger* و *A. flavus* هي السائدة منها بنسب وجود تساوي 33% و20% بالترتيب. أما عزلات فطريات ال (*Aspergillus*) الخضراء، فقد وجدت بشكل رئيس في توابل الدواجن والفلفل الأحمر الحار. أما وجود *A.* fumigates في بعض العينات فله أهمية طبية كون هذا النوع من الفطريات يفرز سموم أخرى تدعى غليوتوكسين. هذا النوع من الفطريات تم الكشف عن وجوده في عينات من السماق، الفلفل الأسود، الزنجبيل والأعشاب الطبية.

لتحديد فطريات ال *Aspergillus* المفرزة للأفلاتوكسينات، تم استخدام ثلاثة طرق بعد زراعة الفطريات على وسائط مختلفة يتم تعريضها لاحقا بعد نمو الفطر لبخار الأمونيا أو الفلوريسنت. بينت طريقة بخار الأمونيا باستخدام الوسيط الغذائية CAM والوسيط الغذائية ADBM أنه من أصل 22 عزلة هناك 19 مفرزة للسموم، أي بنسبة 90.9% من العزلات. أما الطريقة الثالثة باستخدام الفلوريسنت على الوسيط الغذائية CAM، فقد بينت أن 59.1% من العينات فقط تحتوي فطريات مفرزة للسموم.

أما فيما يتعلق بسموم الأفلاتوكسين بي1 في العينات الغذائية المفحوصة بواسطة ELISA، فقد تبين أنه من أصل 116 عينة، هناك 46 تحتوي على سموم بي 1 وذلك بنسب تتراوح بين 0.4-13.5 ميكروغرام/كغم وبمعدل عام يساوي 4.62 ميكروغرام/كغم. المستويات القصوى وجدت في عينات الفلفل الأحمر الحار وتراوحت بين 4.15 و13.5 ميكروغرام/كغم. أما فيما يتعلق بالعينات التي وجد فيها سموم بي1، فقد تبين أن 49% منها (22عينة) تحتوي مستويات بي 1 تفوق الحد المسموح به وهو 5 ميكروغرام/كغم.

أخيرا، فإن تحليل نشاط المياه (water activity) في العينات أظهر أن المعدل العام هو 0.522±0.051. بناء عليه، لم تكن هناك أي علاقة بين مستويات سموم بي 1 وعدد الفطريات الملوث.

وفي الختام تقدم نتائج هذه الدراسة المزيد من الأدلة على ضرورة انتظام مراقبة وجود الافلاتوكسين للأغذية المستوردة وكذلك المحلية .

2. Introduction

The paradox in "poisoning oneself" is not far from the illustration of one adding a dash of aflatoxin-containing black pepper, or a dash of spice to his plate for that matter and can even be compared to a sip of aflatoxins in a mug of hot coffee to summit the effect. Not only does it initiate at farm and end in the spoons of our destiny, but unfortunately, it also finds its way in the forks of our little ones and on the cash in society's pocket. In the scope of this section, many aspects will be covered to illustrate the complex yet simplified analysis of the problem statement of this study, natural occurrence of mycobiota and aflatoxigenic strains and the presence of aflatoxins in foodstuff. The characteristics and nature of this natural and dangerous toxin will be stated in addition to its occurrence in food with reference to its toxicological effects. The nature and occurrence of the responsible fungi will also be affirmed in this section.

2.1. What are Aflatoxins?

Being ubiquitous in a wide range of food and feedstuff, around the World and throughout history, aflatoxins remain quite notorious for many people. Aflatoxins are among the major mycotoxins found in nature and among the most toxic natural products being produced by certain fungi of the genus "*Aspergillus"*. As secondary metabolites, aflatoxins are not needed for the normal growth of fungi nor for their function (Detroy et al., 1971). Hence, their production depends on environmental and developmental factors like moisture, light, temperature and pH (Georgianna et al., 2008). In the chemistry of their structure, aflatoxins are of the coumarin group of compounds where part of the molecule consist of a dihydrofuran moiety merged to the

coumarin substitute (Asao et al., 1963; van Dorp et al., 1963). Figure 1 depicts the chemical structure of one of the many types of naturally occurring aflatoxins, namely Aflatoxin B1 (AFB1). There are 16 types of aflatoxins, where four types are commonly found in contaminated food and feed, namely aflatoxins B1, B2, G1 and G2 (Weidenbörner 2001). These initials (B & G) have been given due to the ability of these types of aflatoxins to fluoresce blue (B1 & B2) and green (G1 & G2) colors under UV light whereas the numbers have been given accordingly to their mobility on Thin Layer Chromatography "TLC" (Bennett and Klich 2003). Other aflatoxins, like M1 and M2, are still considered important hydroxylated metabolites which are bio-transformed varieties and are found to occur in milk and urine (Cole and Cox 1981). The originality of this form allows them to expand to the massive range of dairy products consumed by the typical consumer due to the contaminated feed found in farms and industry in general.

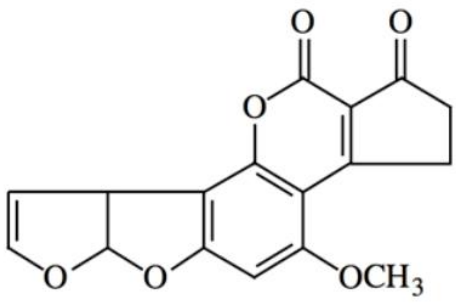


Figure 1. The chemical structure of aflatoxin B1 (Madrigal-bujaidar and Madrigal-santillán 2010).

2.2. Aflatoxins Production

Aflatoxins have been shown throughout studies to be produced by many fungal species. The first fungus that was identified for aflatoxins production was the green mould "*Aspergillus*

flavus". The toxin this species produces was named Aflatoxin (**A:** from ***Aspergillus*; fla:** from ***F****lavus*- **toxin** as a poison**)** (Sargeant et al., 1961). *A. parasiticus* was later found to produce the toxin as well (Codner et al., 1963). These two species are of the most common two food spoiling fungi from the *Aspergillus* genus; section Flavi that are characterized by having brown or yellowish-green shades coloring their conidial heads and dark sclerotia. In addition to these two species, later studies confirmed other aflatoxin-producing species namely, *A. nomius* (Kurtzman et al., 1987), *A. parvisclerotigenus* (Saito and Tsuruta 1993), *A. pseudotamarii* (Ito et al., 2001), *A. bombycis* (Peterson et al., 2001), *A. minisclerotigenes* and *A. arachidicola* (Pildain et al., 2008), *A. ochraceoroseus* (Klich et al., 2000) *A. rambellii* (Frisvad et al., 2005), *Emericella astellata* (Frisvad et al., 2004), *E. venezuelensis* (Frisvad and Samson 2004) and *E. olivicola* (Zalar et al., 2008). These discoveries made the total of aflatoxin-producing fungal species equal to 13 (Varga et al., 2009). Recent updates, however, have increased the count to more than 20 species by adding the following species to the list: *A. pseudonomius, A. pseudocaelatus* (János Varga et al., 2001), *A. togoensis* (Rank et al., 2011), *A. mottae, A. sergii, A. transmontanensis* (Soares et al., 2012), *A. novoparasiticus* (Gonçalves et al., 2012), *Aschersonia coffeae* and *Aschersonia marganita* (Kornsakulkarn et al., 2012; 2013).

Most toxigenic fungal species have been found to produce a consortium of different mycotoxins including different aflatoxin types, not being restricted to one or two types. Producers of aflatoxins B1 and B2 which commonly occur in food samples include *A. flavus, A. parasiticus, A. nomius, A. arachidicola* and *A. minisclerotigenes* and those that commonly occur in food samples and produce aflatoxins G1 and G2 consist of *A. parasiticus, A. nomius, A. arachidicola* and *A. minisclerotigenes* (De Saeger, 2011). Since aflatoxins are formed primarily

by the common *A. flavus* and *A. parasiticus* and being commercially important and widely distributed strains, the green *Aspergillus* species from group Flavi will be monitored in the selected food varieties of this study as differentiating them by morphological markers of pure cultures is complicated.

2.3. Mycotoxigenicity

Mycotoxigenic fungi are those that are able to produce toxins. Fungal species that are found in a large number of genera and individuals from the same species are typically chemoconsistent, meaning that if an isolate from a species is mycotoxigenic and produces, for instance, aflatoxin, then isolates of the same species are also toxigenic. However, that is not the case for all isolates since the silencing of certain metabolite gene clusters as secondary metabolites are influenced by biotic and abiotic factors of the environment (Frisvad et al., 2004; Frisvad and Larsen 2015). Hence, these epigenetic factors shape the toxigenicity of these organisms (Cichewicz 2010). For instance, *A. flavus* will not always produce aflatoxins.

Assessment for mycotoxigenicity of isolates might be a challenging approach under restrictions of equipment and standards. However, testing the aflatoxigenic capability of *Aspergillus* isolates can be done by several methods. The most complicated method includes the PCR systems which detects aflatoxigenic isolates by targeting genes of biosynthetic pathway of aflatoxins (Färber et al., 1997). Conventional methods include screening for *Aspergillus* species in food items using specific medium where then isolates are obtained for further identification and assessment of toxicity by sub-culturing on a specified medium. The medium is then

dissolved in an organic solvent, like chloroform, followed by specific approaches such as HPLC, ELISA or TLC, where aflatoxins are extracted and assayed. Even though reliable, these methods confer to expenditure of time, demanding high cost and intensive lab work. For this reason, employing PCR methods as mentioned previously (Shapira et al., 1996) or RT-PCR (Sweeney, Pàmies, and Dobson 2000) is preferred. In addition, a more direct however, semi-quantitative measurement, makes the use of culture mediums supplemented with influencing compounds. Methylated/beta-cyclodextrin supplemented medium enhances fluoresce of aflatoxins and can be used as a supplement to culture mediums. This approach, developed by Fente et al. (2001), allows the visualization of aflatoxigenic species under long wave UV radiation. Therefore, this method is a qualitative assessment of the presence or absence of fluorescence and thus, aflatoxins. Therefore, a known concentration of spores in a suspension, or even the use of single spore inoculation, is needed (Cotty 1988; Lemke et al., 1989; Degola et al., 2011a;). Similar direct visual approaches include the Czapek-Dox agar supplemented with sodium nitrate and an aqueous extract of groundnuts (de Vogel et al., 1965) and a Czapek's solution agar containing corn steep liquor and lacking a nitrogen source which is termed as the fluorescent APA agar (Hara et al., 1974). Saito & Machida (1999) tested the aflatoxigenic species of *Aspergillus* depending on the change of color of the reverse to pink with ammonia vapor exposure. In addition, a coconut agar medium (Lin 1976) and a modified version by Davis et al. (1987), a silica gel medium by Torrey & Marth (1976), a coconut extract agar containing sodium desoxycholate by Lemke et al. (1989), a coconut extract broth by Lemke et al. (1988) and a coconut cream agar by Dyer & McCammon (1994) were all formulated for the same purpose. The coconut agar medium has gain popularity in literature (Degola et al., 2011a; D'Mello et al., 2014; Ezekiel et al., 2014) and so gained the basis of the aflatoxigenic analysis in the study.

2.4. Conditions for Fungal Growth

In order for microorganisms to grow and obtain positive metabolic activity, certain resources and conditions must be available. Among these is water that is, of no doubt, vital for all life forms. Water activity (a_w) is the amount of "water present in a substance that is free" and not chemically or physically linked to a substance, thus it is available directly to support fungal growth (Megan and Olsen 2000). It quantifies the association between the moisture found in foods and the capability of microorganisms to thrive (Perricone and Gallo 2017). The concept of "water activity" is an intrinsic factor defined as the "ratio of the water vapor pressure of the food to the vapor pressure of pure water at the same temperature" (Berk and Berk 2013). It is a significant variable, which can be adjusted in control and mitigating, processes as part of a risk analysis. Water activity is a dominant, if not major, post-harvest control strategy used in the Hazard Analysis and Critical Control Point (HACCP) systems to prevent food poisoning (Megan and Olsen 2000). On a general scale, most bacteria and fungi grow only at a_w values greater than 0.90. If the a_w value is below 0.8 the only organisms likely to grow are xerophilic fungi and osmophilic yeasts (Roberts and Greenwood 2007). In general, the optimal a_w value for growth is 0.996 (Gqaleni et al., 1997). However, water activity range of 0.80-0.82 supports only minimal fungal growth (Northoltl et al., 1977). Therefore, the moisture content, and specifically water activity, of crops is usually directly associated with mycotoxin contamination (Mora and Lacey 1997).

Temperature is another important factor for fungal growth. In the range of 24°C to 32°C, temperature is deemed optimum for *Aspergillus* growth and aflatoxin B1 production (Gibson et al., 1994). For *A. flavus,* the optimum temperature for growth and the production of aflatoxins is

33°C at a water activity of 0.99. Whereas, for *A. parasiticus*, the values are 35°C and 0.95 for temperature and a_w, respectively (Sanchis and Magan 2004).

Another important factor for fungal growth is the substrate nature. The production of aflatoxins and toxicity was found to be associated significantly with the ratio of carbohydrates-lipids to proteins (Farag et al., 1986). Cereals represent a typical substrate for the growth of *A. flavus* (Pitt 2000) that were first recorded in feed which, consisted mainly of groundnuts (Blount 1961).

2.5. Aflatoxins: Health Implications and Mode of Action

Due to its high toxicity, AFB1 is the most well studied aflatoxin type. It is the most widely distributed toxic type of aflatoxins and is considered the most potent natural carcinogen (Squire 1981; Ellis et al., 1991; Suárez-Bonnet et al., 2013; Ostadrahimi et al., 2014). AFB1 was graded by the International Agency for Research on Cancer (IARC) as a "group I carcinogen" (IARC, 2012). The toxicity spectrum of aflatoxins, and mainly AFB1, is not only restricted to acute or even chronic carcinogenicity and hepatotoxicity, but expands also to teratogenicity, genotoxicity, immunotoxicity causing a syndrome called aflatoxicosis (Groopman et al., 1988; Eaton and Groopman 1994; Bondy and Pestka 2000). In 2012, the number and amount of crude incidence rate of liver Cancer in the State of Palestine was 73 and 1.7, respectively (Mohammadian et al., 2016). Aflatoxins may be a cause of correlation with hepatocellular carcinoma "HCC" incidence or merely one of the factors of causation (Ramirez et al., 2017). The maximum permitted intake of AFB1 in food ranges between 5-20 µg/kg as established by many countries (Madrigal-Santillán et al., 2010).

The mechanism of action of aflatoxin toxicity occurs as the oxidation reaction by liver cytochrome P450 enzymes changes AFB1 to an epoxide that is reactive (8, 9-epoxide; AFBO). This epoxide can covalently bind to guanine of DNA forming adducts (Eaton and Gallagher, 1994) (Figure 2). One of the major and most applicable biomarker for measuring past aflatoxin exposure in blood is the aflatoxin-albumin adduct (AF-alb) which has a half-life of approximately 2 months. Therefore, this adduct allows the assessment of chronic exposures to the toxin (Turner et al., 1998). Other biomarkers, as the aflatoxin-N7-guanine adducts released in the urine, and the hydroxylated metabolite of AFB1 (AFM1) released in breast milk, have been deployed. However, their presence reflects an exposure over a very short period (Wild et al., 1990). In a recent study, a high-resolution mutational spectrum of AFB1was detected in a mouse model, before tumors are induced by aflatoxins by many months (Fedeles et al., 2017).

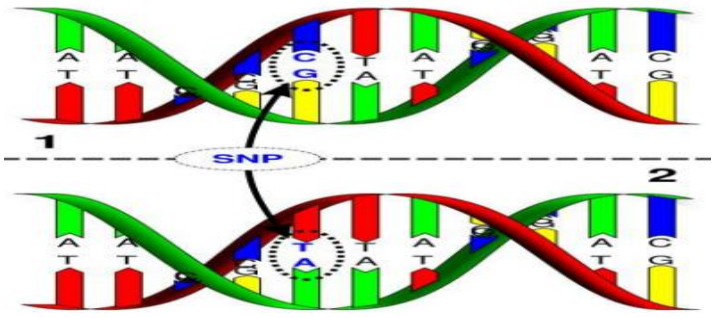


Figure 2. An illustration of the genotoxic effect of aflatoxins by the formation of a Single Nucleotide Polymorphism (SNP) and point mutation of Guanine to Thymine at codon 249 in the p53 gene (Bbosa et al., 2013).

2.6. Aflatoxins Food Contamination: Economic Impacts

Being indicted for the deaths of 100,000 turkeys in Britain, aflatoxins were first recognized in 1961 in feed stuff (Blount 1961). Possible contamination may often occur prior or even post harvesting. Hence, if the harvest was not dried on time and moisture during storage of the commodity was above critical standards, then they will be prone to mold growth and consequently toxin production. In addition, biological factors including the presence of insects or rodents can assist in the mold infestation and toxin production. The production of aflatoxins in commodities may be prevented at temperatures below 20°C and moisture/humidity levels below 14% where water activity should not exceed 0.7 to 0.8 for protected storage of crop (Sanchis and Magan, 2004; Magan and Aldred, 2007). The intricacy of contamination levels of aflatoxins depends on the species of *Aspergillus* and the environmental conditions including temperature, moisture and gas constituents (Magan et al., 2003). However, the complexity of the occurrence of this toxin is not only dependent upon harvesting steps, but also upon storing, processing, and transporting (Trucksess and Scott 2008; Turner et al., 2009). Thus, precautions are to be taken at all levels from the farms to consumers.

Serious economic losses are seen in industrial or agricultural production in which reductions in production efficiency, quality, and yield are accompanied. It was approximated that losses due to aflatoxins contamination, to the US corn industry would range from $52.1 million to $1.68 billion per annum, having considered that aflatoxins contamination in the food supply of the USA is generally trivial (Mitchell et al., 2016). At a regional scale, contamination of local commodities is one level of exposure being food or feed, however a secondary level and yet

potentially higher in risk of exposure is the imported food in the multiple lines of trading that surpass regulation policies.

Aflatoxin-producing fungi have a global distribution and the variety of foods that were reported contaminated includes cereals (corn, rice, wheat, oat, barley...etc), nuts (Peanuts, pistachio, walnuts, hazelnuts ...etc.) and spices (red chili, black pepper, ginger, cardamom, saffron, cinnamon, thyme, anise … etc.) (Goldblatt 1969). In addition, coffee, tea, tobacco, dried fruits, milk and milk products, flour, corn meals, sesame seeds, sunflower seeds, pumpkin seeds, peanut butter, honey and many other main foods were all found to be contaminated with aflatoxin as well (Ellis et al., 1991; Oliveira et al., 2000; Batista 2003; Iamanaka et al., 2007; Fakoor Janati et al., 2012; Hacıbekiroğlu and Kolak 2013; Swaileh and Abdulkhaliq 2013; Tosun and Arslan 2013; Ostadrahimi et al., 2014; Jeswal and Kumar 2015).

2.7. Aflatoxins Analysis Methods

There exists a wide variety of methods for the detection and the quantification of Aflatoxins. Chromatographic methods include "Thin-Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC)". The first two, (TLC and HPLC), are considered as "gold standards" and are so the most broadly applied approaches. Spectroscopic methods are a second general approach, which includes fluorescence spectrophotometry and frontier infrared spectroscopy. The simpler and perhaps cheaper alternatives include immune- chemical approaches, which include chiefly the enzyme-linked immunosorbent assay (ELISA), the radioimmunoassay (RIA), the immunoaffinity column (IAC),

and immunosensors. In the scope of this study, the ELISA method has been chosen for rapid screening purposes of large sample numbers. HPLC offers fast and accurate detection of aflatoxins using high tech machines in operation of experienced technicians (Wacoo et al., 2014). In a research in 2002, three different approaches were compared: ELISA, HPLC and fluorometry, where 14 samples of spiked sesame butters (tahini) were evaluated for precision, accuracy, and straightforward link (Nilufer and Boyacioglu, 2002). It was found that the HPLC and fluorometric methods were highly correlated and effective in recoveries with small deviations for the evaluation of total aflatoxins in samples. Due to deviations in replicates, it was proposed that the ELISA method is suitable as a screening method. Quantification is typically achieved via fluorescence detectors and uncommonly by UV detection (Shephard 2009). Even though aflatoxins are naturally strong fluorescent compounds, it is found that the fluorescence of AFB1 and AFG1 significantly decreases depending on the aqueous mixtures used (Kok 1994).

2.8. Study Area: PALESTINE

Imported commodities passing without regulations jeopardize the general population's health and economy. Furthermore, the distributers and retailers are held responsible for poor storage practices that increase the risk of contamination. This may be seen in Palestine, the center of this study. In Palestine, studies on aflatoxins in foodstuffs are limited. Furthermore, all foodstuffs, imported or locally produced, offered to consumers at local markets are not tested for the presence of aflatoxins. Palestine is considered a subtropical region with a Mediterranean climate that is suitable for fungal growth. Hence, aflatoxigenic fungi are adapted to warm and humid climates as the tropics and sub-tropics of several developing countries (Leong et al.,

2012). Many foodstuffs, like spices and dried fruits that are imported from tropical countries can sustain proper fungal growth.

In the mid area of Palestine, the city of Ramallah is situated, elevating 880m above sea level. This city may be considered to have a buoyant economy containing merchandise from its local neighbors from the north, south, east and west, in addition to international goods from all around the World. Hence, the Palestinian consumers are exposed to a wide spectrum of diverse commodities that may most probably be contaminated.

3. Literature Review

3.1. Mycotoxins: A Global Crisis

The occurrence of mycotoxins in foodstuff is global (Ezz El-Arab et al., 2006). South Asia and South-East Asia are considered areas with the highest occurrence of aflatoxins in foodstuff. Among 17,000 food samples analyzed, 78% of the samples contained detectable amounts of mycotoxins (Schatzmayr and Streit 2013). The devastating outcome of economic losses due to mycotoxins affects approximately 25% of the World's food crops annually (Smith 2001; Bryden 2007). Aflatoxins exposure and the consequent health implications mostly arise in regions of the World with elevated incidence of hepatitis B like China, the sub-Saharan Africa and Southeast Asia (Liu & Wu, 2010). Hence, a risk assessment analysis has estimated at a global scale that about 25% of the hepatocellular carcinoma cases could be attributed to aflatoxins exposure (Liu & Wu, 2010). Worldwide outbreaks of aflatoxicosis, have caused 500 cases with acute illness and 200 cases of deaths (CDC 2004). In 2004 at the rural areas of East Kenya, residents have grown maize contaminated with molds which lead to the most reported outbreaks of aflatoxicosis (Azziz-Baumgartner et al., 2005). In 2004, a survey of global occurrence of mycotoxin prevalence was surveyed in many regions including the Middle East, where specifically aflatoxin prevalence reached to a percentage of 14% (Schatzmayr and Streit 2013).

The limits for aflatoxins contamination types AFB1, AFB2, AFG1 and AFG2 in consumer food products namely “nuts, peanuts, maize flour, figs and their products, and other foods” should be no more than 5µg/kg for AFB1 and 15µg/kg for all four types together (Vincenzi et al., 2011). These levels are to be taken into great consideration and strictly used in control systems of management plans. Aflatoxins are distributed worldwide, and most countries have thus, set standard admissible levels of all aflatoxins which are regulated and specifically assigned for different products of food and feed (Commission of the European Communities, 2003). More than 100 nations have set standards for maximum aflatoxins tolerable levels in food which include regulations on only total aflatoxins, AFB1, or both (Wu and Guclu 2012). The regional FAIR report (Food and Agricultural Import Regulations and Standards) states a set of procedures that are to be implemented on shipments of grains. A quarantine inspector and main regulations in assessing aflatoxins are obligatory requirements where only thereafter the shipment may be permitted for release (Shachar 2010). The consequent of contamination from storage conditions post-importing is thus a matter of discussion, in addition to local products that are produced, stored and sold throughout the region where acceptable measures of mitigation from contamination are to be contended.

3.2. Surveys Around the World

There are vast variations in origins and storage conditions of foodstuff around the World. Studies reporting the presence of aflatoxins in local, as well as, imported foodstuff are common. In Algeria, samples of different commodities, including peanuts, almonds and dried figs, were assessed for the occurrence of aflatoxins. A total of 69 samples (61.6%) were found

contaminated with AFB1 (Ait Mimoune et al., 2018). In Egypt, AFB1, AfB2, and AFM1 were found in 13 out of 79 maize samples (16%), having a maximum level detected at 197.5 µg/kg which is higher than admissible levels especially for a country considered "third" among top maize importers (Abdallah et al., 2017). In Kenya, maize, millet and sorghum were found to be contaminated with AFB1 at percentages of 76%, 64% and 60%, respectively (Sirma et al., 2016). In Turkey, 48 samples of herbal teas were analyzed, in which 43 were reported positive of aflatoxins where the highest reading was for chamomile tea at a concentration of 34.18 µg/kg (Tosun et al., 2016). In Malaysia, 95 packed food products which included, rice-based, wheat-based, corn-based, oats-based, oilseeds, nuts and spices were screened for AFB1 and it was reported that 72.6% of the tested food products were reported positive with *A. flavus* being the dominant occurring species (Reddy et al., 2011). In Brazil, 62 dried fruit samples were screened for *A. flavus* which contaminated 18% of the sultana (raisin) samples and 2% of dried figs, all of which were evaluated as being aflatoxins producers (Iamanaka et al., 2007). In the same study, correspondingly, 3 of 19 (16%) and 11 of 19 (58%) of white sultana and dried figs were found positive of aflatoxins. In Saudi Arabia, 48 green coffee beans were screened and 83% were found positive of AFB1 (Bokhari 2007a). In Morocco, 55 samples of spices including paprika, ginger, cumin, and pepper were analyzed for AfB1 where average contaminations were 2.88, 0.63, 0.03 and 0.09 µg/kg respectively (Zinedine et al., 2006).

Wheat, rice and corn are among the main components of worldwide cuisines, hence, assessment of these heavily consumed foods are critical. This becomes a crisis in third World countries, which depend mainly on one category of food types that might be heavily contaminated. Screening procedures have been applied in countless regions around the World as

awareness of mycotoxicity burgeons. In a study conducted in China, 110 samples were assayed in which 107 samples including maize and dehusked brown rice were found to contain aflatoxins (Liu et al., 2016). In a study in north Iran, ten wheat samples (29.4%) were found contaminated by aflatoxins with the highest measure recording 15 ng/g (Namjoo et al., 2016). The species *A. flavus* and *A. parasiticus* were recently detected on dried corn kernels collected from Vietnam markets (Ly et al., 2015). Rice has been seen to be contaminated at a global scale (Elzupir et al, 2016). Lentils (ElMaraghy 1988) and chickpeas (Ahmad and Singh 1991) were found to be contaminated but not as common and widely surveyed as the previous food items. Nuts and dried fruits are main ingredients in deserts and main dishes in addition to being merely snacks, assessment of aflatoxins are crucial. A study in Pakistan was executed on dry fruits and edible nuts in which 132 out of 307 samples were found contaminated with aflatoxins in peanuts with no shells. The samples included dried plums, dates, dried apricots, raisins, dried figs, almonds, walnut with and without a shell, peanuts with and without shells, watermelon seeds, melon seeds, pistachio with and without a shell, pine nuts and cashew nuts (Masood et al., 2015). Total aflatoxins were reported in 3% of a total of 67 samples Jordan where the mean contamination level was 9.62µg/kg in walnut and rice (Salem and Ahmad 2010). It was reported that of forty eight of coffee bean samples, 83% were found positive of AFB1 (Bokhari 2007a). Countless studies and varieties have been reported and thus, baseline studies and risk assessments are needed.

In Palestine, studies evaluating mycotoxins in foodstuff are limited. In literature, aflatoxin screening was found in an old, unpublished thesis in which fifty single samples of spices and medicinal plants were evaluated in Nablus, northern West Bank (Al-Mosleh, 2002). Analysis was undertaken via an immunoaffinity column (IAC) assay and a spectroflurometer.

Results indicated that 48 of the samples analyzed were found to contain aflatoxins 13 of which containing over 20ppb of aflatoxins. Lisker et al. (1994), studied over 300 groundnut samples were obtained and examined for aflatoxins presence using ELISA. The selected groundnuts were chosen under the categories of exported and locally consumed or sowed products. Aflatoxins were not detected in the samples, however, when samples were stored at high humidity the buildup of aflatoxins were found to be significant seven days after incubation (Lisker et al., 1994). Other unpublished reports in 2010 and 2011 (Barel et al., 2016) analyzed imported grain samples and reported levels of AFB1 in 3% of 536 samples analyzed. A study in 2012 reported an accidental death of 50 dogs due to fatal aflatoxicosis from contaminated commercial diet which contained corn contaminated with 30-300 µg/kg AFB1 (Bruchim et al., 2012). In a screening of *Aspergillus* isolates that produce aflatoxins, 1,626 species of *Aspergillus flavus* were isolated from groundnut seeds and soil samples (Joffe 1969).

3.3. Mycotoxigenic Screening

Determining mycotoxins and mycotoxigenic fungi in food and feed may be considered as an exceptionally extensive complex topic intensively spreading in the literature of science, health and economy since the 1980s. Complications typically lies in the taxonomy of mycotoxigenic fungi and in the instable classifications, particularly when the genera *Aspergillus, Alternaria, Fusarium* and *Penicillium* are considered, in addition to the vast amount of emerging new species. Hence, for this reason a polyphasic classification and identification is generally preferred, despite the lacking in developed standard guides or identification keys, which should base such an approach. It is convenient and recommended to initially, isolate the fungal colonies

under question and identify to genus level by morphological characteristics (De Saeger 2011). In determination if toxigenic isolates cultural and/or analytical approaches are then implemented. Abbas et al. (2004) compared cultural and analytical assessment of 203 isolates of *A. flavus* where TLC was used to confirm three cultural assessments. TLC determined 97% of the isolates positive for toxigenicity and via ELISA, the fluorescence based media containing β-cyclodextrin, the formation of a yellow pigment on the mycelia and reverse of colonies and the formation of a pink color after exposure to ammonium hydroxide vapor reported 93%, 73%, and 70% of the isolates positive, respectively. In a very recent study, a polyphasic approach was used to study the toxigenic ability of *A. flavus* isolates from peanuts where about 58.6% and 68.5% of the isolates produced aflatoxins and cyclopiazonic acid respectively (Norlia et al. 2018). Bokhari (2007a), found *Aspergillus* and *Penicillium* species contaminating 96% and 42%, respectively, of 45 coffee samples where 6 out of 18 *A. flavus* isolates were aflatoxin producers. Mycotoxigenic fungal screening is needed in food mycobiota studies. Applications may include assessment for avoidance of the strain or pinpointing control strategies. The efficacy of atoxigenic strains *of A. flavus* as bio-controlling agent has been studied and implemented (Degola et al., 2011b).

4. Objectives

The present study, therefore aims at evaluating the natural occurrence of mycobiota and aflatoxigenic isolates from the Flavi group of *Aspergillus* in food samples marketed in Palestine. Evaluation will be based on basic cultural media procedures. Food items to be analyzed will be belonging to 8 different main food categories (spices, grains and cereals, nuts and seeds, dried fruit, coffee, herbs/tea, tobacco and mua'ssel, and others). Finally, levels of the most dangerous aflatoxin type (AFB1) will be measured using ELISA.

5. Methodology

5.1. Collection and storage of samples

A total of 229 samples of commonly consumed food products belonging to 8 different food categories were purchased randomly from markets in the West Bank of Palestine during the year 2018 which were either packed or unpacked (Table 1). The total number of samples were partitioned depending on analysis (Figure 3) .

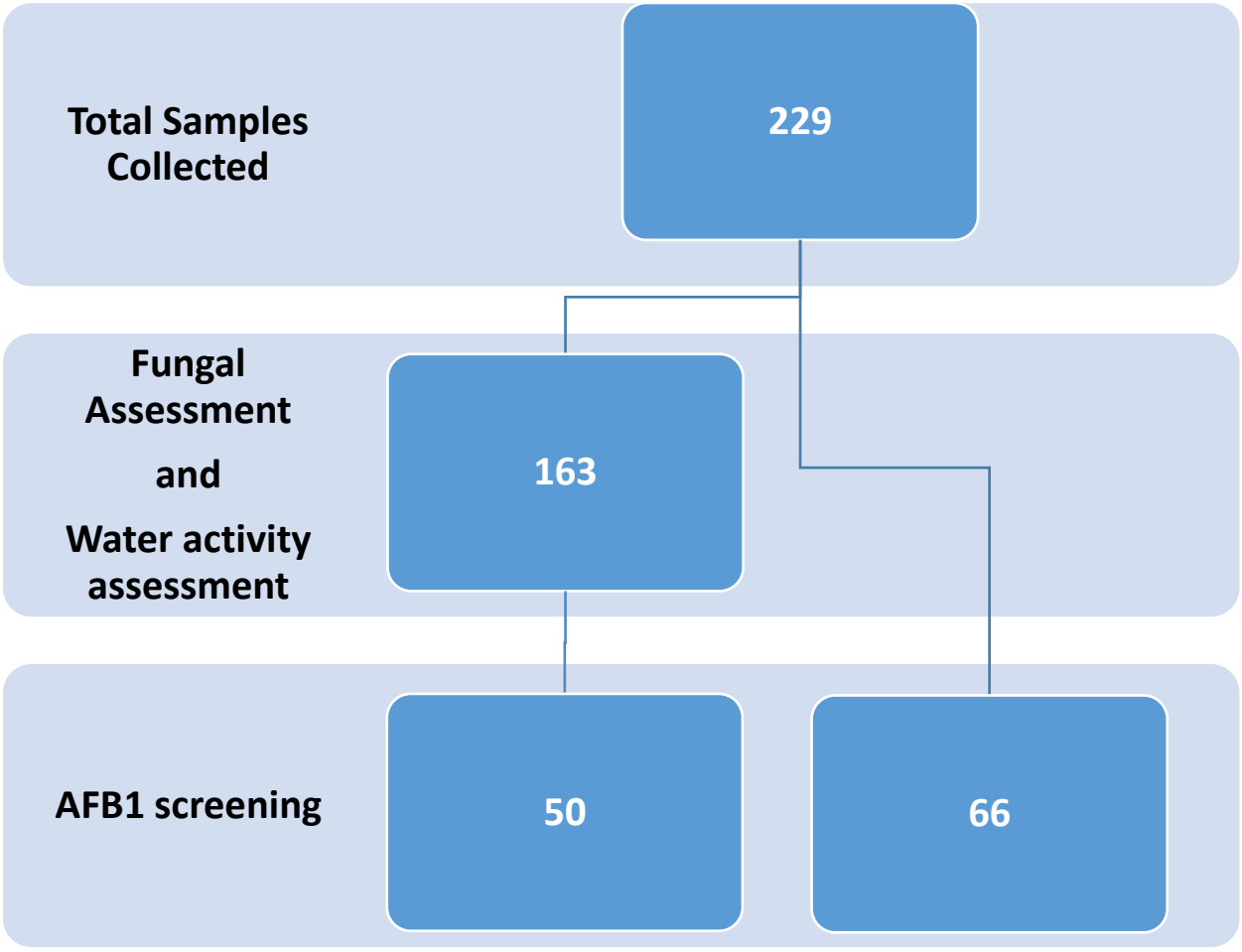


Figure 3. Summary of sample analysis

Table 1. Summary of food samples collected from markets in Palestine during the present study.

Category	Food Type	Total #
Coffee		**10**
	Coffee Beans Dark Roasted	3
	Filter Coffee	3
	Instant Coffee	2
	Coffee Beans Light Roasted	2
Dried Fruit		**14**
	Apricot	3
	Dates	3
	Figs	4
	Raisins	4
Grains and Cereals		**25**
	Chick Peas	4
	Corn	3
	Corn Flakes	3
	Flour	3
	Lentils	4
	Lentils Orange	3
	Rice	2
	Wheat	3
Nuts and Seeds (Roasted)		**23**
	Almonds	3
	Cashew	3
	Peanuts (without shell)	3
	Pistachio (without shell)	3
	Sesame Seeds	4
	Sunflower Seeds	4

	Watermelon Seeds	3
Others		**15**
	Chocolate Spread	3
	Cocoa	3
	Corn Oil	6
	Peanut Butter	3
Spices (Ground)		**118**
	Cardamom	16
	Chicken Spices	17
	Red Chili	15
	Ginger	16
	Mixed Spices	19
	Black Pepper	18
	Sumac	17
Teas and Herbs		**9**
	Black tea	3
	Green Tea	3
	Mixed Herbs	3
Tobacco		**15**
	Tobacco	12
	Mu'assel	3
Grand Total		**229**

Samples were collected from Ramallah District (31°54′N 35°12′E), but included local samples from different geographic regions and imported ones from different countries (Figure 4).

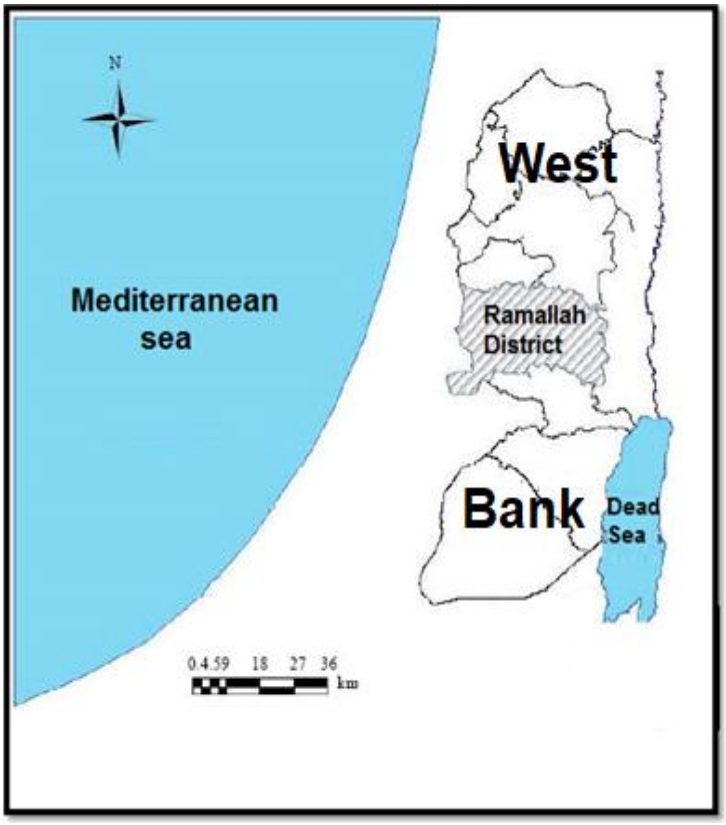


Figure 4. Geographical map of the sampling area in Ramallah District in the West Bank of Palestine.

All samples purchased weighed around 135 to 1000g depending on the nature of packaging by retailers. Each sample was well mixed by hand; thereafter 55-75 grams were weighed and divided into subsamples as follows: 5 grams for fungal analysis, 10 grams for water activity analysis and 40-60 grams for mycotoxin analysis.

All samples (except spices, flour and dried fruits) were properly stored in labeled sealed containers kept in a dark, dry closet at room temperature. Spices, flour and dried fruits were stored in a refrigerator at 4°C to prevent any further fungal growth and mycotoxin production prior analysis.

5. 2. Water activity determinations

The water activity (a_w) of all samples were determined by automatic analysis, using AwTherm, ROTRONIC (Rotronic AG, Bassersdorf, Switzerland) under the standard of ISO 21807 in which samples were stabilized to room temperature of approximately 25±1 °C. Samples were weighed to 5 or 10grams and placed in a plastic container which was situated in the equipment chamber for equilibration. Finally, after around 5-15 minutes when the change between readings was no more than 0.01 aw unit, water activity was recorded.

5. 3. Fungal screening

5. 3. 1. Preparation Of Food Samples

Food samples were processed under standard aseptic conditions to prevent external contamination. The yeast and mold isolation method was based on that of Reddy et al. (2009 and 2011). In sterile containers, food samples were accurately weighed and thereafter suspended, either as 5 g of solid sample or 5 mL of semi-solid sample, in peptone (0.1%) water of pH 7.2 adjusting the final volume to 50 mL with an initial dilution of 1:10. Thereafter, mixture was shaken for 5 min using an Orbital Shaker (IKA™ KS 260basic) and allowed to settle for 2-3 min before the wash liquid was serially diluted in peptone water (0.1%) in preparation for plating on the proper agar.

5.3.2. Fungal Enumeration In Food

To culture fungi, Rose Bengal Potato Dextrose Agar "R-PDA" was used. It is an agar used for enumeration of fungi and is composed of an infusion from Potatoes (200 g/l), Dextrose (20.00 g/l) and Agar (15.00 g/l) with the addition of Rose Bengal (Bokhari 2007a). The preparation steps were according manufacturer's recommendations as follows: From the PDA, 39.0 g were suspended in 1000 ml of distilled water and supplemented with Chloramphenicol (0.050 g/l) to prevent bacterial contamination and Rose Bengal dye (25mg/l) (King et al., 1979) to retard the growth of the fast growing fungi. The suspensions of agar were autoclaved at 121º C for 15 minutes. Thereafter, it was poured in Petri dishes and left to cool down to room temperature before use.

Using the dilution plate method (Beuchat 2003), 1ml of the homogenized sample was aseptically pipetted into a sterile glass tube, containing 9ml of 0.1% peptone water, ensuing a dilution of 1/100. The same dilution procedure was repeated to get serial dilutions of 1/1000 and 1/10000 in duplicates. Thereafter, 0.2ml of each diluted food suspension was added to the pre-poured plates containing the proper medium. After properly spreading the sample on a rotary plater with a bent glass rod spreader, the culture was incubated at 25 ± 2°C and examined at day 3 to count fungal colonies before overgrowth of heavily contaminated samples. Final counting was done on day 7 leaving the cultures untouched or carefully handled to prevent overestimation of colony forming units (CFUs) due to the fragile nature of fungus which can form satellite colonies (Ganguli and Deshmukh 2007). Petri dishes containing R-PDA and 1ml of sterile peptone water were used as blank negative controls.

Visual examination took account of morphological characteristics of fungal colonies and microscopic features which were then identified using the available taxonomic keys (Larone, 2002; Malloch, 2017). Microscopic images of fungi were prepared by the scotch tape method using lactophenol cotton blue (Larone 2002). Images were professionally taken via Moticam X camera (Motic ®, USA).

After incubation for 7 days at the temperature of 25-28°C in a dark incubator, the plates were examined using a standard colony counter (BOECO, CC1) for presence of yeasts and filamentous molds. Countable plates of 8.5cm Petri dishes, those presenting 15 to 150 fungal colonies, were enumerated (Deák and Beuchat 1996). The results were expressed in colony forming unit per gram or milliliters of sample (CFU/g or ml) by using the equation below(Silva et al. 2012):

$$\mathbf{N= \sum C/ V \times 1.1 \times d}$$

N: number of colonies/ml or g

$\sum C$: sum of all colonies on the two dishes counted from two successive dilutions

***V*:** is the volume of inoculum placed in each dish (in milliliters)

***d*:** is the first dilution retained

The frequency (F) of genus isolation was calculated as indicated in Ghiasian et al., (2004) by using the following equation:

F = (number of samples contaminated with a genus/total number of samples) x 100.

And the Incidence(%) was calculated by

I = (Number of grains infected by a genus / Total number of grains) ×100

5.3.3. Aflatoxigenic Screening Of Isolates

Green *Aspergillus* isolates from food samples were recovered for pure cultures by sub-culturing in Petri dishes containing PDA supplemented with chloramphenicol at a concentration of 0.025mg/l. After 7 days of incubation at 25 ˚C in a dark incubator, cultures were examined for purity of isolate. The isolates were then sub-cultured again in separate sterile glass tubes containing PDA slanted agar for 7 days at a temperature of 25˚C in the dark, which were then maintained in sterile mineral oil, tightly sealed with a screw cap under aseptic conditions and finally stored at −20˚C.

Two media were used to identify aflatoxigenic isolates of *Aspergillus* and included the *Aspergillus* Differentiation Base Medium "ADBM" and the Coconut Agar Medium "CAM". ADBM is used to differentiate aflatoxigenic *A. flavus* from other species of *Aspergillus* (Bothast and Fennell 1974; Salkin and Gordon 1975). It consists of 10% peptic digest of animal tissue, 20% of yeast extract 0.05% of ferric ammonium citrate, 0.002g/l of dichloran, and 15% agar. The suspensions of agar were prepared as specified by manufacturer and autoclaved at 121º C for 15 minutes. Chloramphenicol was added at a concentration of 0.025mg/l aseptically. Thereafter, it was poured in Petri dishes and left to cool down to room temperature before use.

For the preparation of the second medium, CAM, Davis et al. (1987) procedure was followed with slight modification. Briefly 100 g of shredded coconut was placed in 300ml of hot distilled water and homogenized for 5min. The homogenate was filtered through four layers of cheesecloth, and the pH of the clear filtrate was adjusted to pH 7 with 2N NaOH.

0.2% of Agar was added and the volume was completed to 1L and the mixture was boiled, cooled and then autoclaved for 15 minutes at 121º C. The suspension was cooled to about 40 to 45°C and chloramphenicol was added at a concentration of 0.025mg/l aseptically where then the medium was poured into Petri dishes and left to cool down to room temperature before use.

A loop of inoculum, mycelia and spore mass, was transferred in duplicates to freshly prepared ADBM and CAM under sterile conditions and incubated in the dark at 28°C for 3 days. The plates containing ADBM were recorded for presence of an orange color on the reverse of the colony and plates with CAM were observed upside down on a UV transilluminator for recording fluorescence ability among isolates as positive or negative. In addition, the CAM cultures were also exposed to ammonia vapor by adding one drop (~300µl) of ammonium hydroxide (25%) (Saito and Machida 1999). The formation of a pink color was immediately observed and recorded as positive.

5.4. Quantitative Assessment of Aflatoxin B1 using ELISA

To analyze the natural occurrence of AFB1 in food samples, ELISA kits (HELICA, CA-USA) were used for rapid quantitative screening of AFB1. The ELISA kits purchased for screening were Low matrix AFB1 “981BAFL01LM-96”. The total number of food samples analyzed was 116.

Representative food samples were weighed and some ground to powder using a typical coffee grinder (Moulinex, LM2420, France). Ground samples were passed through a 20mesh (0.8mm) screen until 80% of the sample passed through the screen or ground to a paste. Then

20g of the sample was weighed and placed in approximately 100mL of 80% methanol. Thereafter, all samples were allowed to settle for 2-3 minutes before filtering the supernatant using a Whatman no. 1 fluted filter paper. Finally, 100µL of the filtrate were diluted in 900 µL of the buffer supplied by the manufacturer of the ELISA kit. For ELISA, 100µL of 6 standard solutions, of concentrations 0.02, 0.05, 0.1, 0.2, and 0.5ppm, and 100µL of prepared samples were added to the mixing wells with 200µL of sample diluents. After that, mixtures were transferred (in duplicates) to antibody coated wells and incubated in for 30mins. After automatically washing wells three times with buffer (350 µL) using a microplate washer (Unilab 3000), 100µL of conjugate (Aflatoxin-HRP) were added and incubated in the dark for a further 30 minutes. The wells were then washed again three times, a substrate solution was added, and the mixture incubated at room temperature for 10min. Finally, 100µL of stop solution were added and the intensity of the resulting color / optical density (OD) was read and recorded at the wavelength of 450nm using a microplate reader (Unilab 6000). The absorbance values were divided by that of the 1st standard (0ng/ml) and multiplied by 100% to obtain the percentage of maximal absorbance (maximal binding B_0). An inversely proportional relationship between absorption intensity and AFB1 concentration in the samples was observed in which a logistic-log graph was used to construct a standard dose-response curve. The AFB1 concentration in each sample was calculated by interpolation taking into consideration the dilution factors. The limit of detection (LOD) for the ELISA kits AFB1 was 0.285 ng/mL and the limit of quantification (LOQ) was 0.865 ng/ml.

5 .5. Statistical analysis

All of the data was expressed as means ± standard deviation (SD) unless otherwise indicated. The data was analyzed by using Microsoft EXCEL 2007 with XLSTAT in which limits of detection and quantification and the Pearson correlation was formulated and calculated. The Student t-test was also used to determine significant differences in comparable measures (duplicates) where values of $p < 0.05$ were considered significantly different.

6. Results and Discussion

6. 1. Occurrence of Fungi in Food Samples:

Out of 163 food items assessed for fungal contamination, 157 (96.3%) were found to be contaminated with mold and yeast. Figure 5 shows the consortium of fungal species found in one food sample.

Figure 5. Fungal Diversity found in Mixed Herbs: A 7-day old culture grown on R-PDA medium and incubated at 28°C.

Table 2 summarizes the average counts of fungi in different food categories analyzed. The average count of yeast and molds in all samples analyzed was 1.0 ±0.4 x10^5 CFUs/g with the maximum CFU recorded for dried apricots (5.9 x10^6 CFUs/g) due to the high occurrence of yeast found in the sample. Next in rank were two chili spices and a local tobacco sample, which

all recorded CFUs of 2.0 x10^6 /g of sample. The highest CFUs count recorded by category was for dry fruit category accounting for an average of 4.8 x10^5 CFUs/g of samples. Dried fruit naturally contain a slight higher percent of water content, which is observed in the water activity compared to that of the other categories (Table 2). This is in addition to the high carbohydrate content of this category that enhances the growth of microorganisms. With regard to the average number of CFUs, food categories can be arranged as follows: dried fruits> tobacco> spices> coffee> grains & cereals> nuts and seeds> teas and herbs> others. Results indicated that 31 food samples out of 163 (13%) went beyond the maximum admissible limit of 1 × 10^4 CFUs/g (EC 2006). Minimum counts were calculated and approximated to 400 CFUs/g(Silva et al. 2012).

Table 2. Summary of Colony Forming Units (CFUs/g) in food categories indicating the maximum, minimums and average water activity.

Food Category	Number of samples	Colony Forming Units (CFUs/g)			a_w
		Mean±SEM	Max	Min	
Coffee	10	60800±39414	400000	0	0.331
Dried Fruit	14	483496±419132	5910000	400	0.611
Grains and Cereals	25	32791±16329	400000	400	0.496
Nuts and Seeds	23	17684±13214	300000	400	0.410
Teas and Herbs	9	6144±895	9890	400	0.483
Tobacco	9	247950±220083	2000000	400	0.594
Spices	61	89665±48336	2500000	0	0.526
Others	12	725±437	5500	0	0.367
Grand Average	-	**100421±42253**	-	-	**0.490**

Dilution plating method was found consequent and successful as CFUs were found in decreasing order. In addition, duplicates were not significantly different comparing CFUs of the same dilution throughout samples ($p>0.05$) (Figure 6).

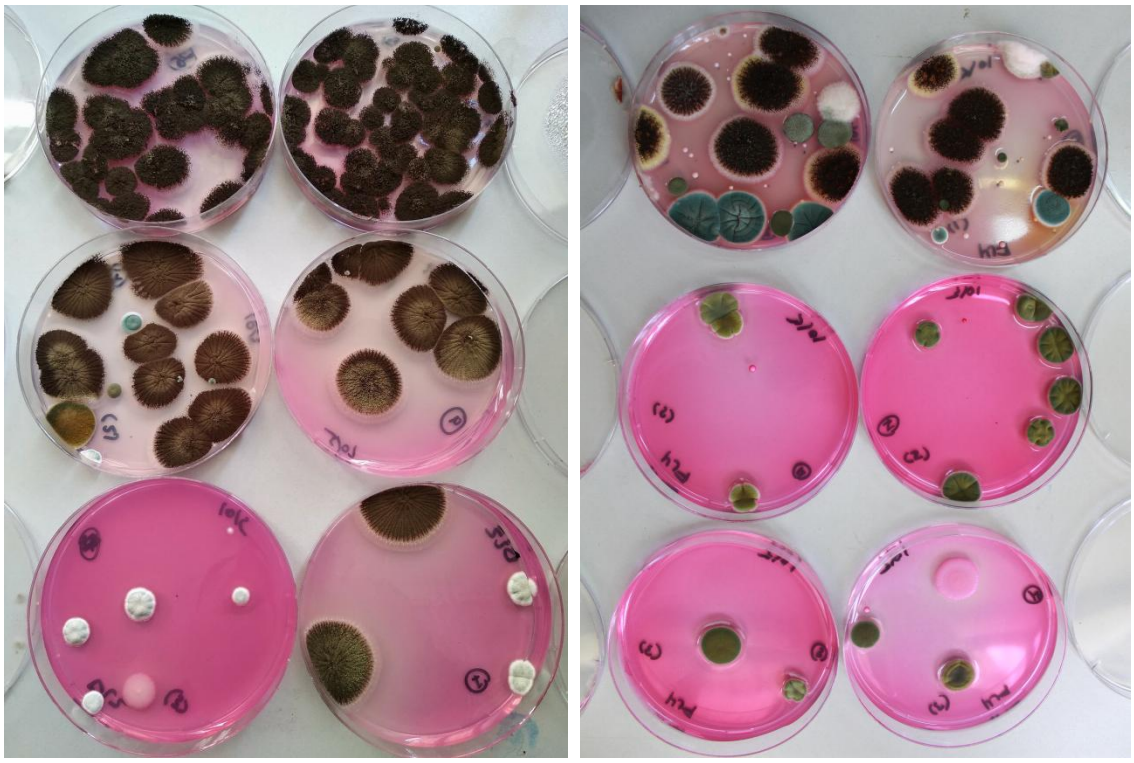


Figure 6. Dilution plate method. Colony culture of two food types incubated for 7 days on R-PDA at 28°C.

In total, 8 genera were isolated using PDA medium and identified according to Malloch (2017) and Larone (2002). *Aspergillus* spp were the most common, contaminating 69.3% of food samples analyzed. *Cladosporium* spp., *Penicillium* spp, Yeast, *Rhizopus* spp, and *Mucor* spp followed in percentages of 35.0, 23.3, 16.6, 16.6 and 6.7%, respectively (Figure 7). Rare spp of *Alternaria* and *Trichoderm* compromised 3.7% of the total fungal occurrence in food samples. In

addition, 31.3% of the species found were categorized under unknown, as they were unable to be identified by basic morphology. Figure 8 displays the typical colonies and microscopic morphology of these genera obtained on PDA medium. In a study that assessed 50 samples of 10 different spices from Saudi Arabia, 15 genera and 31 species of fungi were isolated having the most common genera as *Aspergillus*, *Penicillium* and *Fusarium* (Bokhari 2007b).

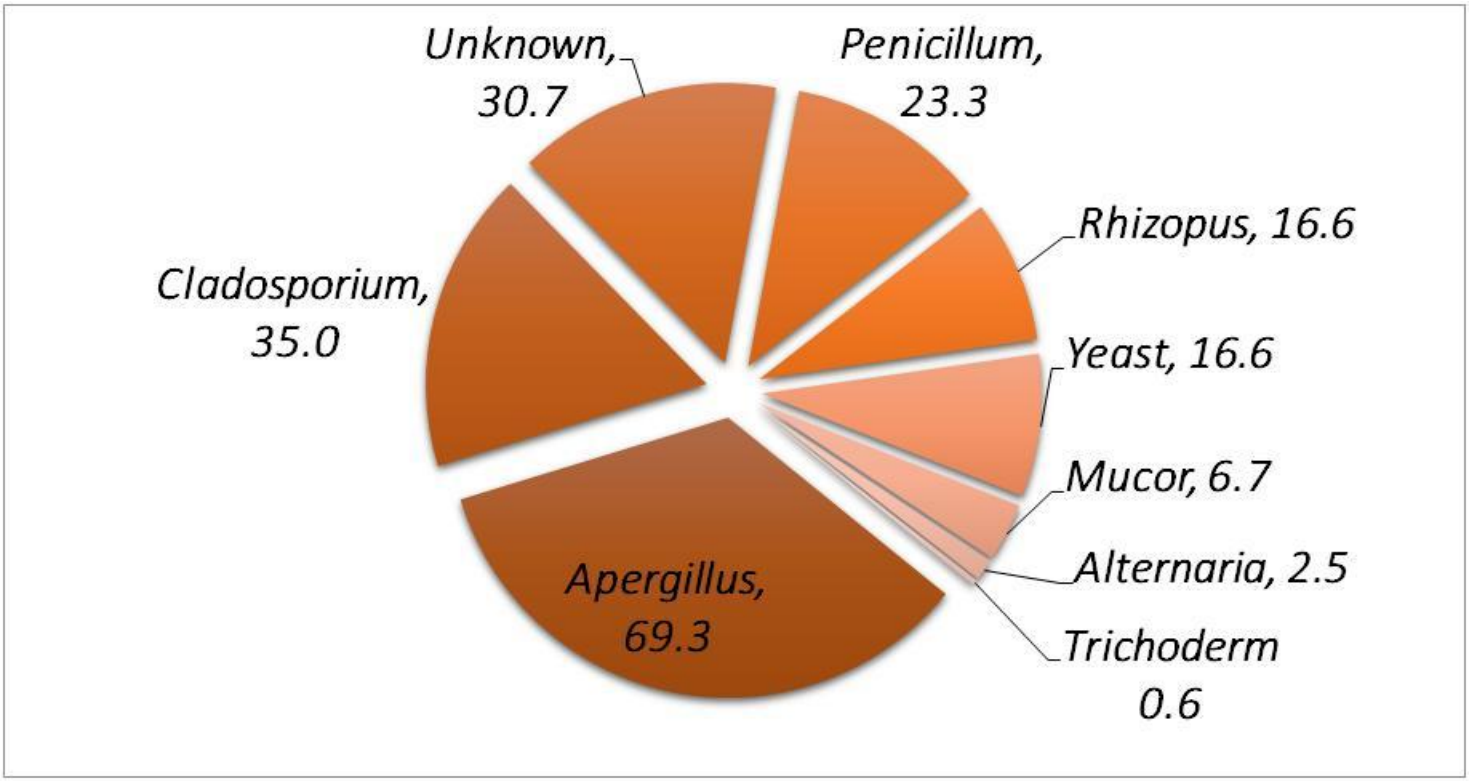
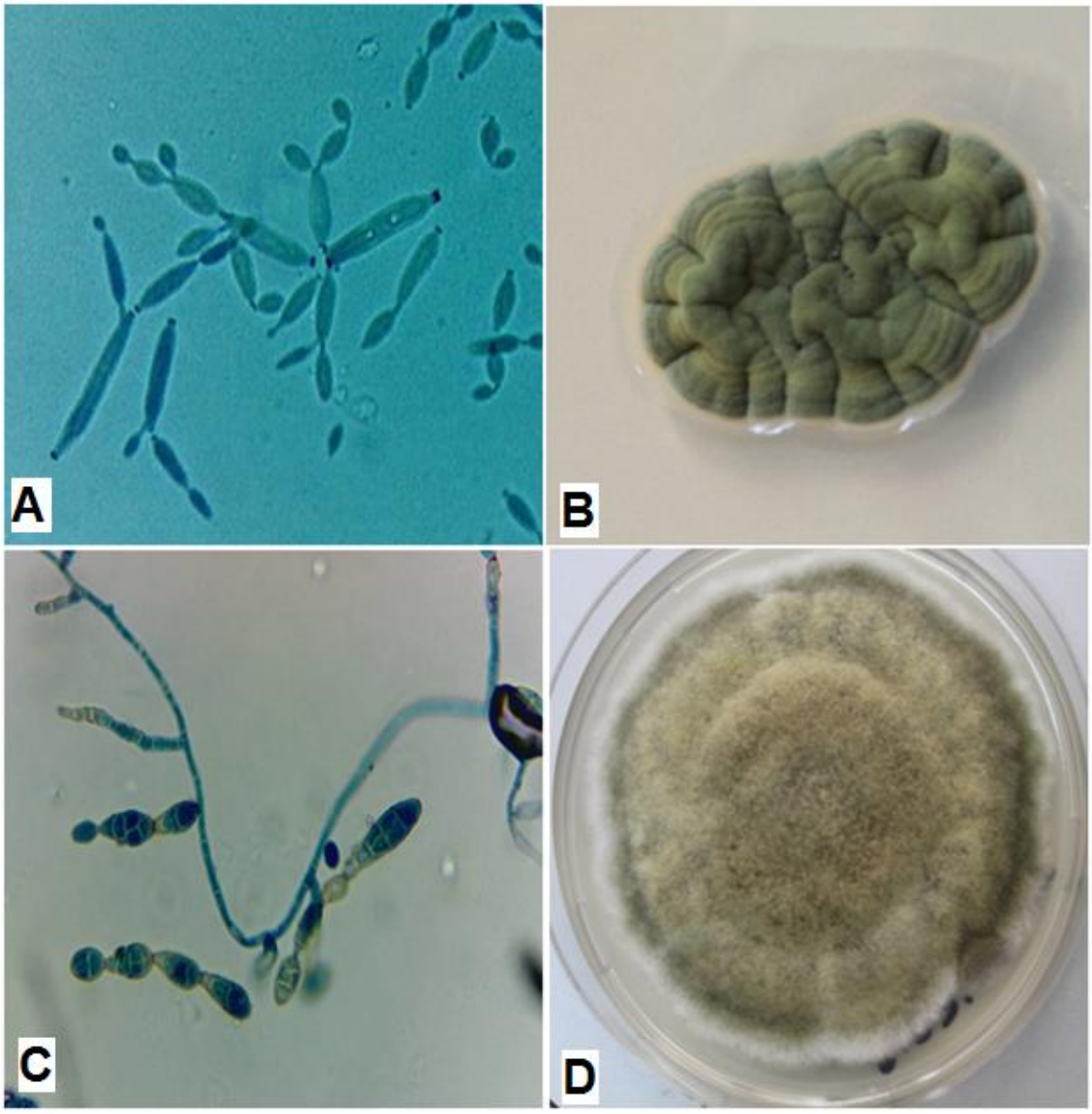


Figure 7. Fungal occurrence in food samples (163) collected from markets of the West Bank, Palestine.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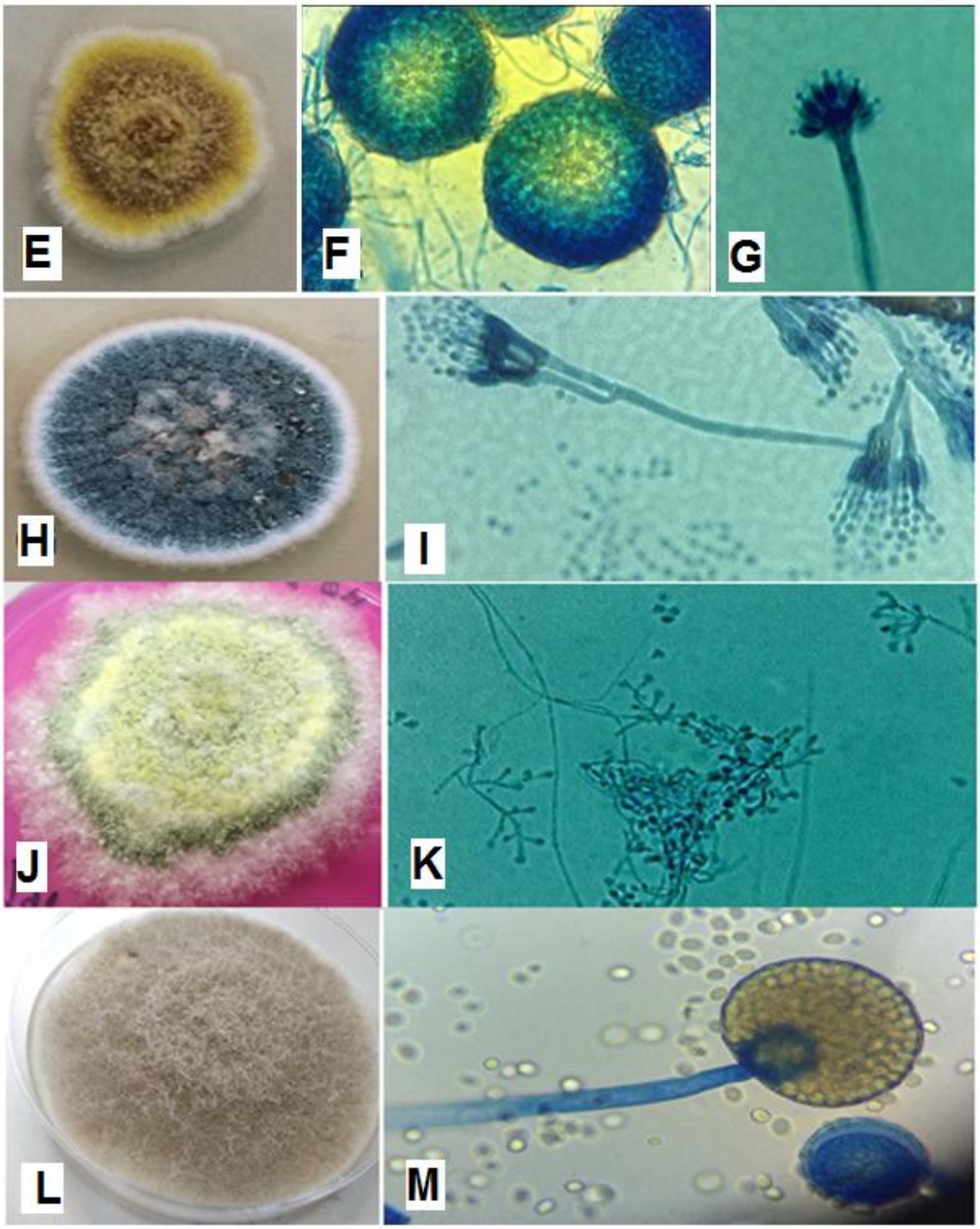
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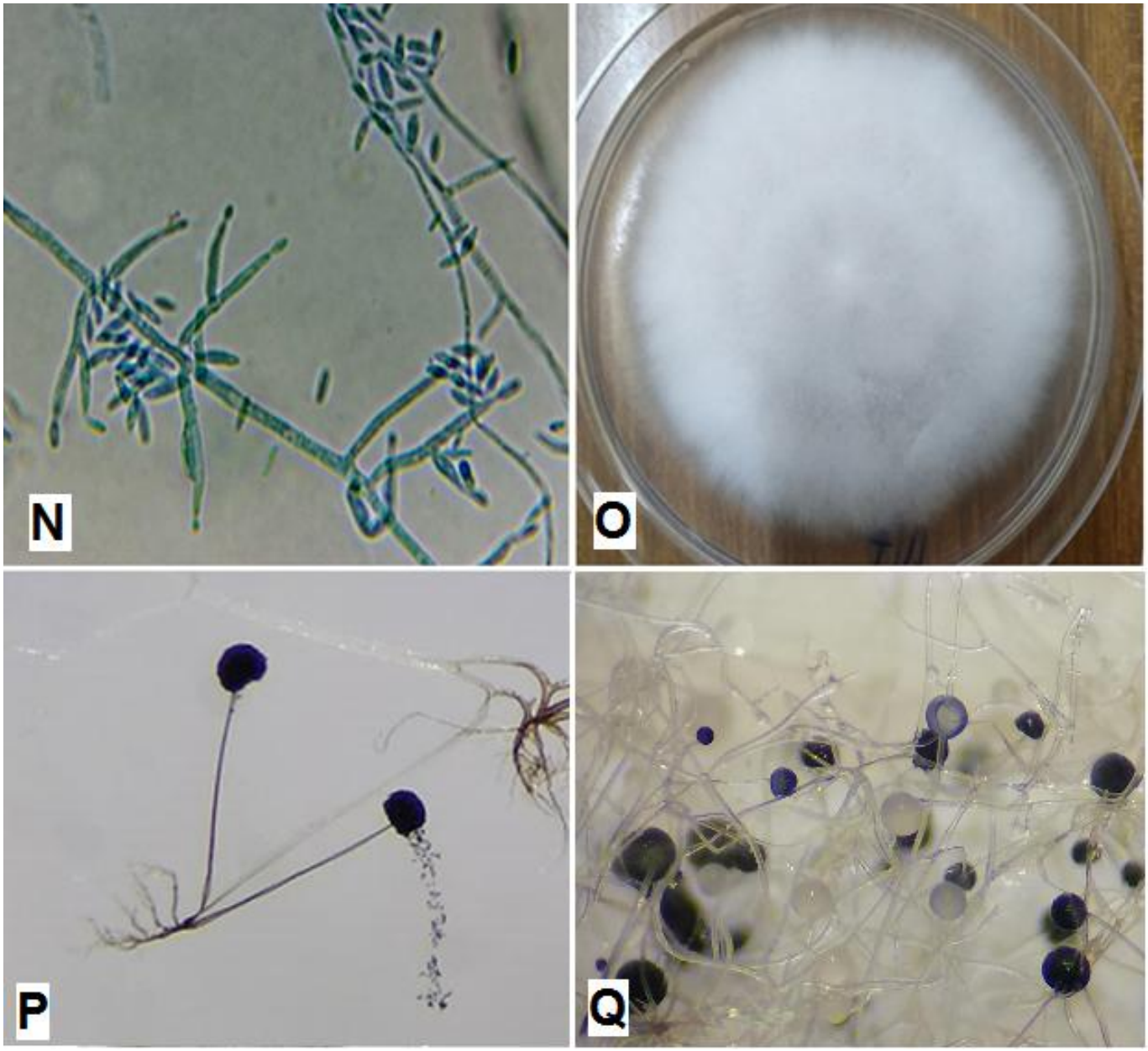

Figure 8: Microscopic and macroscopic morphology of several isolates from different genera. All microscopic images were taken at the magnification of x400 and fungal colony cultures are grown on R-/PDA for 5 -7days. (A+B) *Cladosporium* (C+D) *Alternaria* (E-G) *A. glaucus* with cleistothecia (F); (H+I) *Penicillium*; (J+K) *Trichoderm*; (L+M) *Mucor*; (N+O) *Fusarium*; (P+Q) *Rhizopus*.

By category, grains and cereals were found to be frequently contaminated with *Aspergillus* species where 92% of the samples were found infested. In a decreasing order 84% of spices, 78% of tobacco, 71% of dried fruit, 56% of tea and herbs, 50% of coffee, 48% of nuts and seeds, and 8% of other uncategorized samples were contaminated with *Aspergillus* species (Table 3).

Table 3. Summary of genera incidence (%) in food categories indicating the average number of species found within samples of same category.

	Genera Average	**Genera Incidence**								
Category (number of samples)	Mean±SD	*Aspergillus*	*Penicillium*	*Rhizopus*	*Alternaria*	*Cladosporium*	*Unknown*	*Mucor*	Yeast	*Trichoderm*
Grains And Cereals (25)	3.9±1.9	92	44	16	0	60	44	4	20	0
Spices(61)	3.4±1.7	84	31	34	0	21	25	13	18	2
Tobacco (9)	2.6±1.4	78	11	0	0	67	44	0	0	0
Dried Fruit (14)	4.5±2.1	71	7	7	21	100	71	14	43	0
Tea and Herbs (9)	2.8±2.4	56	22	0	0	11	33	0	11	0
Coffee(10)	1.1±1.3	50	0	0	0	0	40	0	10	0
Nuts and seeds (23)	2.0±1.6	48	13	4	0	26	9	0	13	0
Others (12)	1.0±0.8	8	8	0	0	17	17	0	0	0

From the genus *Aspergillus*, *A. niger* followed by *A. flavus*, (Figure 9), were the dominating species that were found in 33% and 20% of the samples, respectively. Jeswal and Kumar (2015) reported that in all spices from India assessed, *A. flavus* and *A. niger* were the most dominant. In

a study on wine grapes from Lebanon, 550 fungal isolates were recovered where 89.1% of the isolates were *Aspergillus spp* among which *A. niger, A. flavus, A. japonicus* and *A. carbonarius* had the percentages of 81.4%, 12.7%, 1.8% and 4.1% respectively. The remaining 10.9% were belonging to *Penicillium spp* (El Khoury et al., 2006).

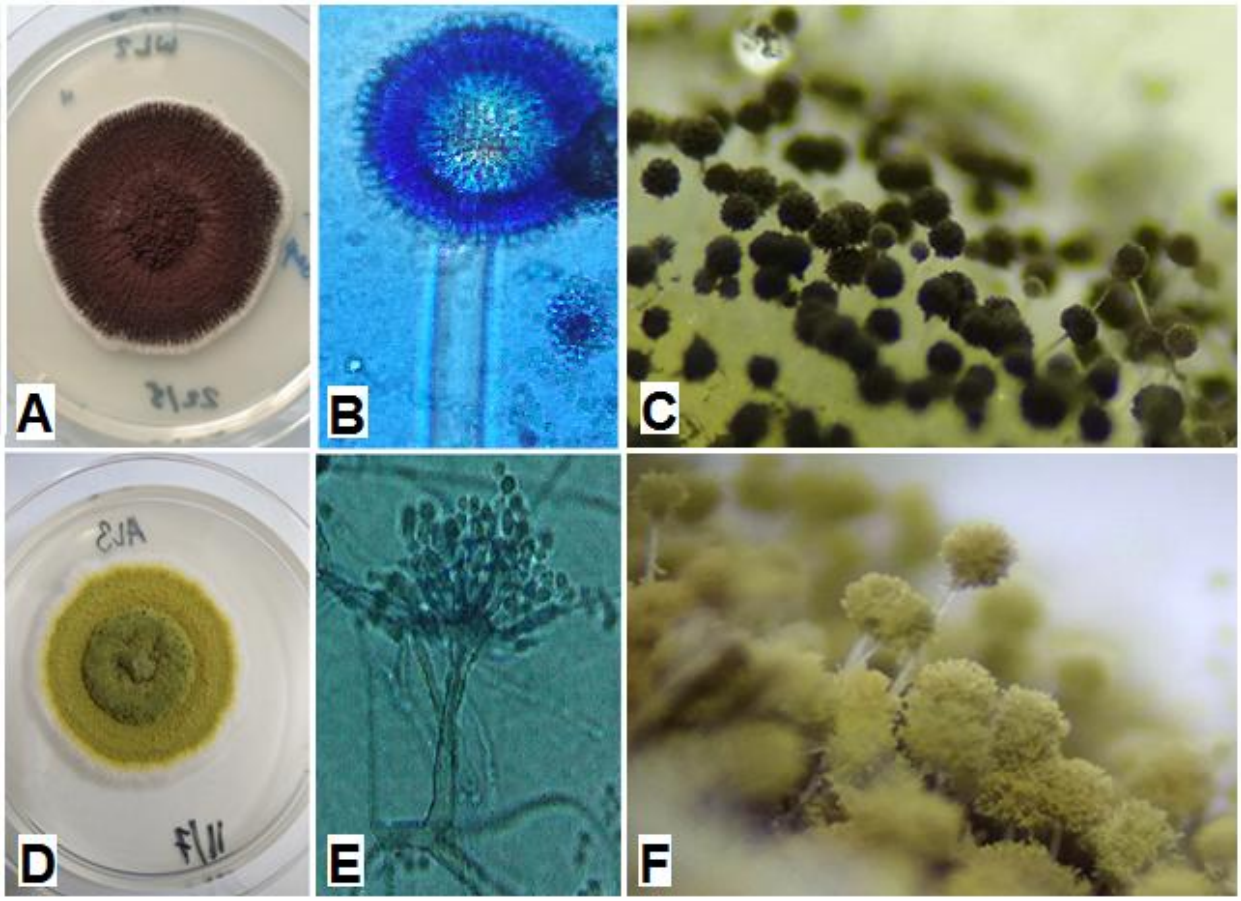

Figure 9. Macroscopic and microscopic morphology of *A. niger* and *A. flavus*. A 7-day old colony grown on PDA at 28°C, microscopic images (400X) of conidiophores and 3D image under a scanning light microscope of *A. niger* (A+B+C) and *A. flavus* (D+E+F).

6.2. Recovery of *Aspergillus* Isolates

In the present study, 14 different food items were found contaminated with green *Aspergillus* spp. adding up to 31 isolates. The food items that were found to be contaminated with *Aspergillus* spp. include chicken spices, red chili powder, dried corn, ground ginger, lentils, mixed spices, ground pepper, almonds, cardamom, dried fig, mixed herbs, peanuts, pistachio, sesame seeds and wheat (Table 4).

Table 4. Origin and percentage of green *Aspergillus* isolates found in food samples analyzed.

Sample #	Sample type	Number of samples tested	Number of isolates found	Percentage to total isolates (%)
1	Chicken spices	12	6	19.4
2	Chili	14	5	16.1
3	Corn	4	3	9.7
4	Ginger	9	3	9.7
5	Lentil	4	3	9.7
6	Mixed Spices	6	2	6.5
7	Pepper	7	2	6.5
8	Almonds	3	1	3.2
9	Cardamom	7	1	3.2
10	Fig	4	1	3.2
11	Mixed Herbs	3	1	3.2
12	Peanuts	3	1	3.2
13	Sesame Seeds	4	1	3.2
14	Wheat	3	1	3.2

In this study, the green *Aspergillus* were found mostly in chicken spices and red chili powder constituting 19% and 16% of the total isolates from these 2 spices, correspondingly. In another study on Pakistani chili samples, only *A. flavus* colonies were observed (Paterson 2007). Whereas in another study from Malaysia, *A. flavus, A. niger,* and *A. tamarii* were observed in all chili samples analyzed and *A. flavus* and *A. niger* were found dominant in other spices investigated (Reddy et al., 2011). *Aspergillus niger, A. flavus, Penicillium citrinum* and *P. pinophilum* were reported as the dominant fungi found in about 300 ground peanut samples analyzed (Lisker et al., 1994). In Egypt, 20 samples of lentil seeds were tested for fungal contamination and results revealed contamination with *Aspergillus* species including *A. fumigatus, A. niger, A. flavus, and A. terreus* (El-Maraghy, 1988).

A. niger which produces another mycotoxin, namely ochratoxin A, has been given a safe status known by the USFDA, as only 3-10% of the strains were toxigenic for ochratoxin A (Schuster et al., 2002). However, in addition to mycotoxins, an important observation seen in this study was the presence of *A. fumigatus* (Figure 10) in some of the food samples and mainly in spices, like black pepper, mixed spices, cardamom, sumac and ginger. Being a significant human pathogen that produces gliotoxin, *A. fumigatus* was considered in this fungal assessment as an additional risk factor.

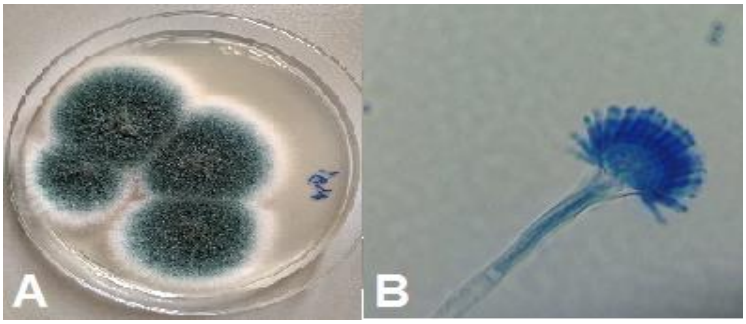

Figure 10: Macroscopic and microscopic morphology of A. fumigatus. (A) A 5-day old colony of A. fumigates grown on PDA at 28°C. (B) Microscopic image (400X) of A. fumigatus conidiophore.

6.3. Assessment of Aflatoxigenicity

When found in suitable environmental conditions, *A. flavus* strains are able to produce aflatoxins and thrive at growing and sporulating (Reddy et al., 2009). Of the green *Aspergillus* isolates, 22 were successfully recovered and further processed to confirm identification of the aflatoxin producing *Aspergillus* species by colony reverse color formation on ADBM (Beuchat, 1984). This medium facilitates a rapid screening method to evaluate food spoilage and differentiating between *Aspergillus* species in general (Bothast and Fennell 1974). Of the isolated species, 90.9% were identified with a bright orange reverse color on the ADBM inferring the isolates being aflatoxigenic compared to the atoxigenic species as seen in Figure 11.

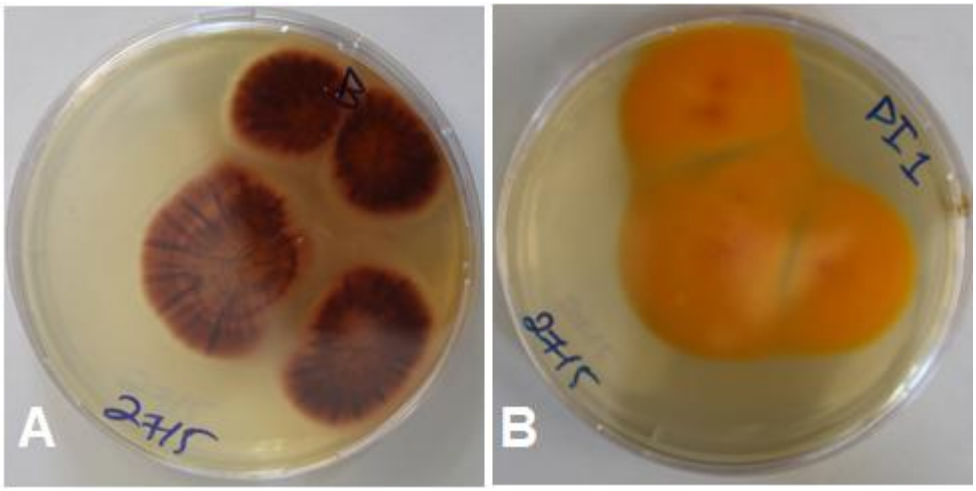

Figure 11. Two fungal strains grown on ADBM for 5 days at 28°C. Atoxigenic *Aspergillus* spp. (A) and Aflatoxigenic spp. (B); the orange pigmentation characterizes aflatoxin producing species.

After growing isolates on CAM, the fluorescence-based assay was used where colonies were exposed to UV light (365 nm) (Figure 12, A & B). The atoxigenic isolates (A) are distinguished by the aflatoxigenic isolate (B), by not displaying a characteristic beige ring around

the colony (Figure 12; A & B). Of the total *Aspergillus* isolates assessed for aflatoxigenicity on coconut agar medium (CAM) by this method, 13 (59.1%) were found to produce aflatoxins (Table 5). In the same Figure 12 (C and D), the ammonia vapor test displays the formation of the characteristic pink color for the indication of the aflatoxigenic strain (D) and no color change for the atoxigenic strain (C). When toxigenicity of the 22 isolates was assessed by ammonia vapor (AV) test, 20 (90.9%) isolates were found to be toxigenic (Table 5). A percentage equal to that of the ADBM results however with small differences in several samples. Some isolates that showed negative toxigenicity by the FB method, gave a weak pink color in the latter method. Few isolates that showed negative toxigenicity by the AV method gave a positive or negative result in the ADBM medium. Three weak samples provided by the AV assay were confirmed positive by the ADBM medium. This might indicate higher sensitivity of the ADBM medium to detect aflatoxigenicity. In addition this confirms the need in using molecular methods like PCR would be more precise at screening for aflatoxigenic species (Manonmani et al., 2005). Table 5 summarizes results of aflatoxigenicity tests for the 22 isolates of *Aspergillus* recovered in the present study.

Riba et al. (2010) found that only 45 isolates (30%) of *A. flavus* obtained from wheat-based products were aflatoxigenic despite the confirmation of 72% by HPLC analysis. From 43 *A. flavus* isolates obtained from maize and soil, 23% were found to display fluorescence on CAM (Thathana et al., 2017). Another study showed that 65% of the *A. flavus* isolates from maize were found to be aflatoxigenic through fluorescence on CAM (Okoth et al., 2012). The *A. flavus* isolates in this study, were analyzed by the ADBM medium and CAM medium with FB procedure and the AV assay and results displayed general agreement.

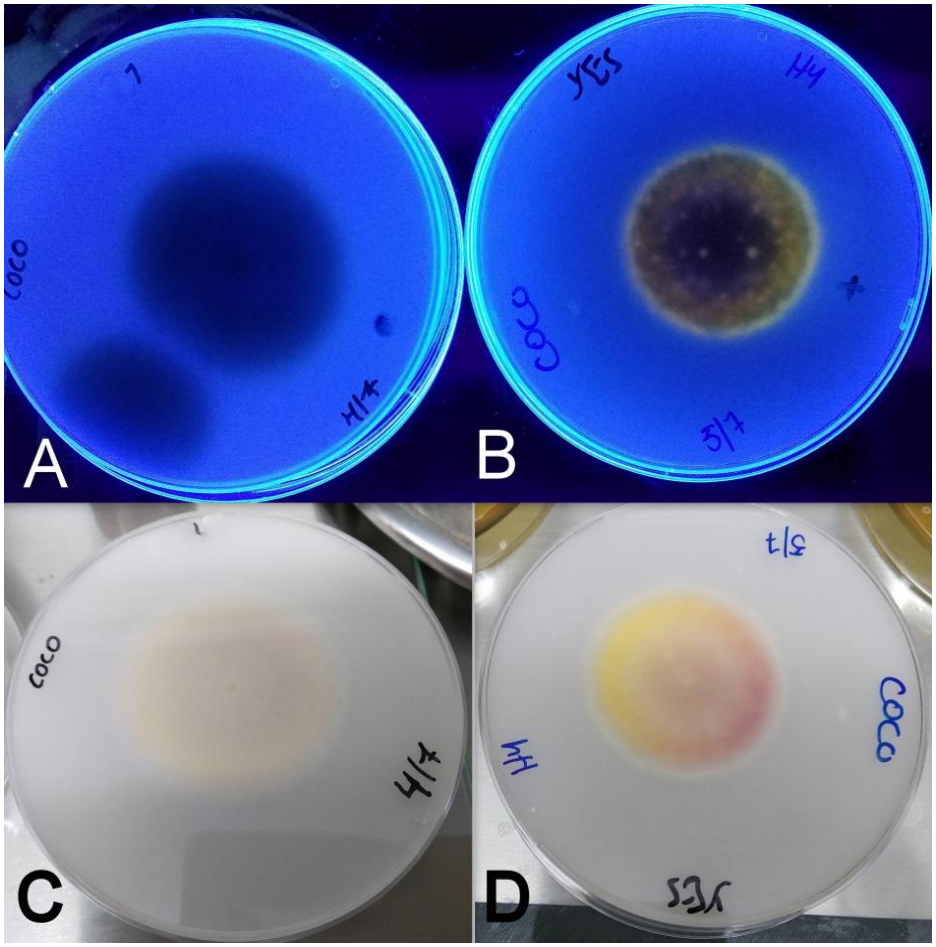

Figure 12. Aflatoxigenic assessment on CAM; isolates were grown on CAM at 28°C for 5 days. (A) and (B) show the Fluorescence based assay displaying the atoxigenic isolate (A) and the characteristic beige ring on the aflatoxigenic isolate (B). (C) and (D) represent the ammonia vapor test displaying the formation of the characteristic pink color for the indication of the atoxigenic strain (C) and aflatoxigenic strain (D).

This supports a study that evaluated the toxigenicity of 524 isolates of *A. flavus*, in which false negatives reached 13 to 15% using the FB assay on different media, in comparison to the AV assay which gave no false negatives on CAM (Fani et al., 2014). The FB analysis on CAM was used as a preliminary step in an evaluation of aflatoxigenic and atoxigenic strains that were capable of interfering with aflatoxin production when grown as co-inoculates in a culture (Degola et al., 2011a).

Table 5. Aflatoxigenic assessment of *A. flavus* isolates. Isolates were assessed in duplicates on CAM at 28°C for 5days. (-) negative for aflatoxin production, (+) positive for aflatoxin production and (W) weak aflatoxin production. (BDL)Below detection limit. AFB1 represents level of Aflatoxin B1 in the original food sample from which isolate was obtained.

Isolate #	Food type	ADBM	FB assay	AV assay	AFB1 (µg/kg)
1	Chili 1	+	-	+	13.5
2	Chili 2	+	+	+	12.4
3	Chicken Spices 1	-	-	W	8.0
4	Cardamom	+	+	+	6.2
5	Ginger 1	+	+	+	1.8
6	Ginger 1	-	-	-	1.8
8	Wheat	+	+	+	BDL
9	Mixed spices	+	+	+	BDL
10	Mixed herbs 1	+	+	+	BDL
11	Pistachio	+	+	+	BDL
12	Ginger 3	+	+	+	BDL
13	Coffee	+	-	W	BDL
14	Sunflower Seeds 1	+	+	+	BDL
15	Sunflower seeds 2	+	+	+	BDL
16	Corn	+	+	+	BDL
17	Chicken spices 2	+	+	+	BDL
18	Lentil 1	+	+	+	BDL
19	Flour	+	-	-	BDL
20	Mixed herbs 2	+	-	W	BDL
21	Almonds	+	-	W	BDL
22	Lentils 2	+	-	+	BDL
	Percentage %	90.9	59.1%	90.9%	27.3%

In the present study, the isolates found positive for aflatoxigenicity originated from red chili, chicken spices, cardamom, ginger, wheat, mixed herbs, mixed spices, dark coffee, pistachio, sunflower seeds, sesame seeds, corn and lentil. Five out of 22 (23%) of the

aflatoxigenic species recovered from food samples had shown positive results of AFB1 production with contents ranging between 1.8 and 13.5µg/kg sample. The incidence of aflatoxigenic *A. flavus* strains in the food items mentioned above with average water activity of 0.47±0.07 indicating no specificity to nature of substrate in the assayed food items.

Of the 18 isolates of *A. flavus* isolated from coffee beans from Brazil, one third produced aflatoxins B1 & B2 (Batista et al., 2003). Aflatoxigenic species were found at higher incidences in peanuts reaching 69% and at lower incidences in wheat and soybeans being 13% and 5%, respectively (Vaamonde et al., 2003). In this study, a red chili powder sample, which was found to contain the highest amount of Aflatoxin B1, at a level of 13.5µg/kg, was evident in containing a toxic strain of *A. flavus.* These results support the concept of supposedly aflatoxigenic species are diverse in the production of secondary metabolites (Frisvad and Larsen 2015). In addition, despite the low levels or even absence of AFB1 in assessed food samples, the presence of the toxigenic strain is quite enough to set a risk for further contamination at intolerable levels under conditions of improper storage of food. This is perhaps the first report from the West Bank, assessing aflatoxin production by isolated *A. flavus* strains from local foods products in Palestine.

6.4. Aflatoxin levels in Foodstuff

Improper storage conditions and lengthened exposure to dust and environmental pollution are poor hygiene practices at typical open markets causing contamination risks of food products. In the present study, 116 samples were screened for AFB1 contamination and the results of the positive samples including percentages, mean of contaminations, and the range contamination for each food category and item are presented in Table 6. Concentrations of the food samples were calculated by extrapolation of values from the regression formula of a 4-parameter logistic regression; $y= 0.133+ (0.991- 0.133/ 1+(x/ 0.087)^{1.965})$. The linearity ($R^2$=0.9987) of the five concentration levels of AFB1 standard solutions for three consecutive analyses was in a good fit of values in the regression curve (Figure 13).

Table 6. AFB1 levels measured in µg/kg sample for 116 samples of spices oils and tobacco.

Category	Item	Number of Samples	Number of Positives	Percentage (%)	AFB1(µg/kg)	
					Mean± SD	Range
Spices		103	39	37.9	5.20±3.54	0.57-13.5
	Cardamom	13	5	38.5	3.85±2.56	1.14-6.20
	Chicken Spices	14	9	64.3	5.52±2.06	0.69-8.10
	Chili	13	11	84.6	8.88±3.09	0.93-13.5
	Ginger	14	6	42.9	1.88±1.82	0.74-5.30
	Mixed Spices	17	3	17.6	3.92±2.93	1.69-6.90
	Pepper	17	3	17.6	1.17±0.72	0.42-2.00
	Sumac	15	2	13.3	4.85±2.55	1.80-6.70
Tobacco		7	4	57.1	1.23±0.86	0.43-2.30
Oil		6	3	50.0	1.62±0.95	0.55-2.60
Total		116	46	39.7	4.62±3.55	0.40 -13.50

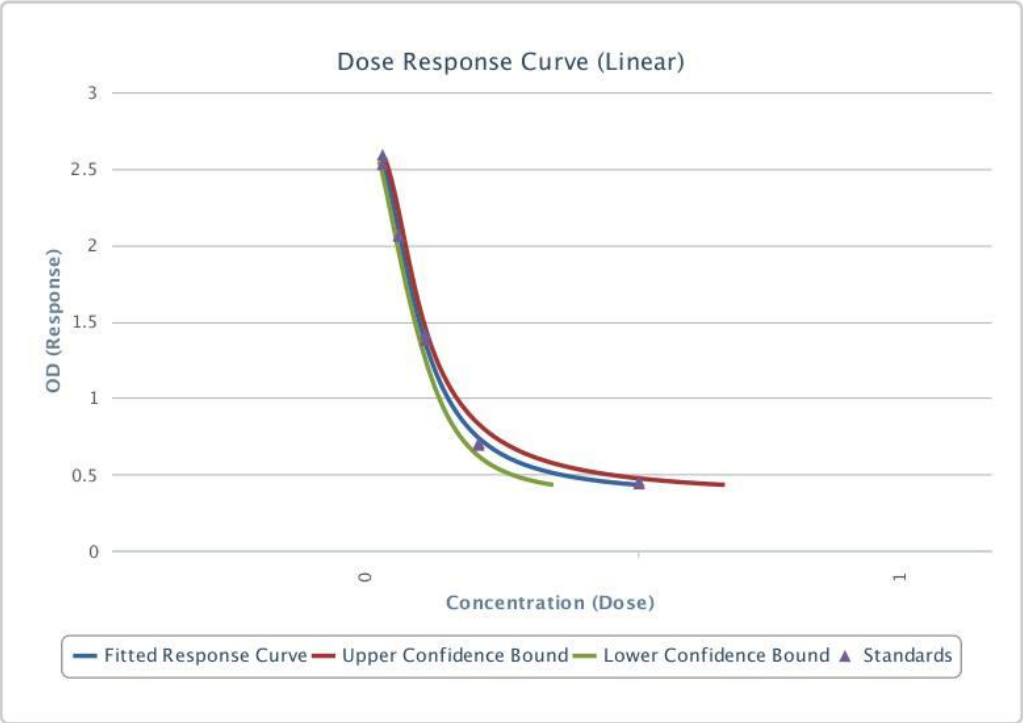

Figure 13. The Dose response curve of ELISA: Effect of AFB1 standard concentration on absorbance at wavelength of 450nm where a 4-parameter logistic regression is formulated with linearity (R2=0.9987) for five concentration levels AFB1 standard solutions.

Of the 116 samples analyzed, 46 (39.7%) were positive for AFB1 and ranged from 0.4 - 13.5 µg/kg. For the positive values assessed, the mean of AFB1 contamination was found to be 4.62±3.55µg/kg. Variation of AFB1 levels were observed among categories and between samples. The highest record of AFB1 was found in chili samples ranging from 0.93 to 13.5µg/kg with the highest mean of 8.88±3.095µg/kg. It also held the highest number of contaminated samples making up 84.6% incidence of contamination in chili samples. Second in rank was highest in chicken spices ranging from 0.69-8.10µg/kg with a mean of 5.52±2.06 µg/kg (Table

6.5). Unfortunate for consumer and retailer 47.8% (22 samples) of the samples were found above permissible limits of 5 µg/kg. Tobacco and oil categories did show contamination of AFB1 however within permissible limits (1.23±0.86 and 1.62±0.95µg/kg, respectively).

In an Irish study, a sample of chili powder recorded the highest concentration of aflatoxin reaching 27.5 µg/kg (O' Riordan and Wilkinson 2008). In a study in Turkey, 58 of 93 organic spices were found positive for AFB1 with 41 samples found above the admissible limits (Tosun and Arslan 2013). In another study, 105 samples of spices were assayed where red chili flake, red chili powder, black pepper and cumin samples were found positive of aflatoxin reaching percentages of 79.2, 63.6, 30.4 and 21.1% , respectively (Ozbey and Kabak 2012). Khazaeli et al. (2017) found that 30.8% of 120 analyzed samples were tainted with aflatoxins and at ranges of AFB1 between 0.7-57.5 µg/kg, fairly exceeding safe limits. Sun drying is a common practice utilized in post harvesting where food commodity and especially chili peppers may be placed on a material distributed one layer above soil (Iqbal et al., 2011). Thus this may be a reason why these samples may be heavily contaminated in addition to poor storage.

Variations among samples, categories and studies may also relate to the antifungal activity of some samples especially those containing essential oils. This is seen in studies like Elgayyar et al. (2001) and Omidbeygi et al. (2007) especially for chili powder (Shantha, 1999; Thirumala-Devi et al., 2000). It is also worth noticing that 18% of the contaminated spices assessed in the present study originated from the same local retailer indicating the poor handling, sanitation and prolonged storage of the food commodities from this unconventional retail store.

Nonetheless, it is important to mention that many samples especially in the case of mixed spices, black pepper and sumac samples extracts passing through screening tests as clear of aflatoxins, hence there are cases of co-extractive, which requires confirmation procedures and through numerous recovery assessments a method for extraction is validated (Senyuva and Gilbert 2011). In a study assessing spices the average contaminations of AFB1in black pepper, ginger, red paprika and cumin were 0.09, 0.63, 2.88 and 0.03 µg/kg, respectively with the highest record owing to red paprika reaching 9.68 µg/kg (Zinedine et al., 2006) .

6.5. Correlation analysis

This may be the first report in correlating, however for the sake of questioning, the relation between the colony counts in the sample, water activity and aflatoxin levels. A Pearson correlation test was formulated for the positive samples analyzed which includes the total of 40/46 samples as two samples were considered as major significant outliers via the Dixon test where $p< 0.0001$. A summary of the observations of all three are described in Table 7 where the means and ranges are indicated. Due to the nature of the food items selected, water activity were within a narrow range with an average of 0.522±0.051. Hence, the correlation was assumed to be at a smaller interval. Figure 14 depicts a matrix of scatter plots and histograms as part of the Pearson correlation test. No significant correlation was found between the three variables tested (fungal contamination levels (CFUs/g), water activity and the AFB1 levels in food sample). This result showing no correlation may not find the causation of contamination however, it may rule out possibilities of contamination. Despite the fact that a larger amount of samples may have been needed to widen the range of possible occurrence at different water activity values, the conclusion may have been more solid and comprehensible. One may infer to the possibility of a

previous contamination not due to the subsisting numbers of colony forming units found in a sample. Another assumption may refer to the presence of atoxigenic fungi since only 27% of the 22 isolates tested for toxigenicity originated from previously contaminated food items.

Table 7: AFB1 levels measured in µg/kg sample for 42 samples of spices oils and tobacco.

Item	No.	CFUs/g		a_w		AFB1	
		Mean±SEM	Range	Mean± SEM	Range	Mean± SEM	Range
Oil	3	1833±1833	0-5500	0.473±0.007	0.462-0.486	1.6±0.5	0.7-2.6
Cardamom	4	32063±23714	4250-103000	0.495±0.007	0.476-0.506	4.7±1.1	1.6-6.2
Chicken Spices	9	6238±1093	400-10300	0.528±0.010	0.473-0.565	5.5±0.7	2.2-8.1
Chili	11	146800±140336	0-1550000	0.508±0.009	0.458-0.583	8.9±0.9	4.2-13.5
Ginger	6	3699±1170	400-6602	0.517±0.013	0.471-0.572	1.9±0.7	0.4-5.3
Mixed Spices	3	12667±7429	4500-27500	0.507±0.013	0.488-0.531	3.9±1.7	1.1-6.9
Pepper	3	5417±961	4000-7250	0.533±0.021	0.492-0.559	1.2±0.4	0.7-2.0
Sumac	2	400±0	400-400	0.703±0.012	0.691-0.715	4.9±1.8	3.1-6.7
Tobacco	1	400±0	400-400	0.536±0.000	0.536-0.536	0.7±0.0	0.7-0.7

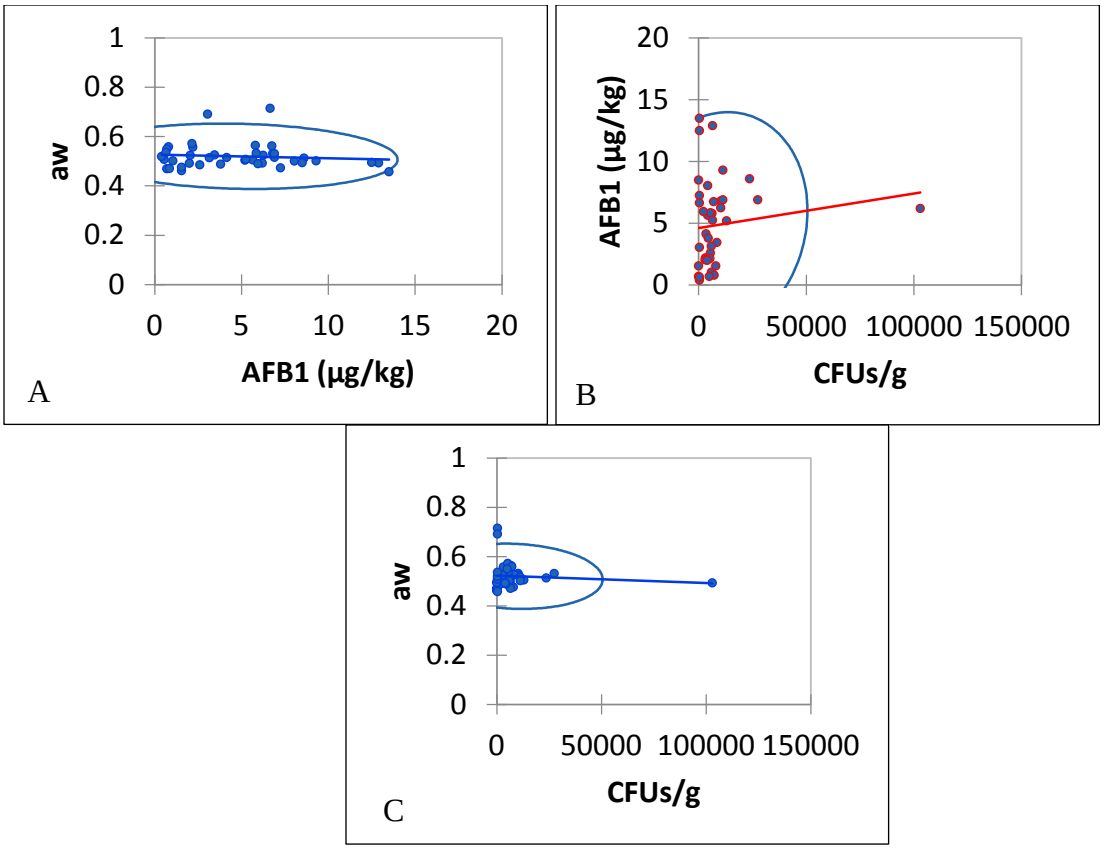

Figure 14. Correlation analysis data. The Pearson correlation analysis was calculated to evaluate the link fungal contamination levels (CFUs/g), water activity and the AFB1 levels in food sample. Correlations made were between aw vs. AFB1 (µg/kg) (A), AFB1 (µg/kg) vs. CFUs/g (B) and aw vs. CFUs/g (C).

Table 8. Correlation analysis data. Correlations made between aw vs. AFB1 (µg/kg) (A), AFB1 (µg/kg) vs. CFUs/g (B) and aw vs. CFUs/g (C) showing calculated, Correlating Variables, Pearson's Correlation (r), Pearson's Coefficient of determination (R2), Confidence Interval 95% and p-values where p< 0.05 is statistically significant. There was no correlation between the three variables.

Designated letter	Correlating Variables	Pearson's Correlation (r)	Pearson's Coefficient of determination (R^2)	Confidence Interval 95%	p-values
A	a_w vs. AFB1 (µg/kg)	-0.102	0.010	]-0.397 , 0.213[	0.528
B	AFB1 (µg/kg) vs. CFUs/g	0.129	0.017	]-0.186 , 0.420[	0.422
C	a_w vs. CFUs/g	-0.095	0.009	]-0.391, 0.219[	0.556

7. Conclusions

After collecting and analyzing 229 food samples belonging to 8 food categories marketed in Palestine, it can be concluded that:

1) 96.3% of the samples analyzed for fungal contamination (total 163) were found to be contaminated with fungi with a mean count of 1.0 ±0.4 x10^5 CFUs/g.
2) Fungal species isolated and identified were belonging to the following 8 genera: *Aspergillus, Cladosporium, Penicilium*, Yeast, *Rhizopus*, *Mucor*, *Alternaria*, and *Trichoderm.*
3) 69.3% of the species occurred in the genus *Aspergillus* being the most frequently observed.
4) The food samples with the highest number of fungal colonies were chili powder (2.5x10^6 CFUs/g) and tobacco (2.0x10^6 CFUs/g).
5) By food category, grains and cereals were found to be highest in *Aspergillus* contamination (92% of samples), followed by spices (84%), tobacco (78%), dried fruits (71%), tea and herbs (56%), coffee (50%), 48% nuts and seeds (48%), and uncategorized samples (8%).
6) *Aspergillus niger* followed by *A. flavus* were dominating *Aspergillus* species in food samples occurring in 33% and 20%, respectively.
7) Of the 22 tested isolates of *Aspergillus*, 90.9% were identified as aflatoxin-producers.
8) Natural occurrence of AFB1 was detected in 46 food samples (39.6%) out of 116 assessed.

9) Levels of AFB1 ranged between 0.40 and 13.5 µg/kg with a mean value of 4.62 µg/kg.

10) Maximum AFB1 levels were detected in red chili powder ranging from 4.15 to 13.5 µg/kg.

11) Of the assayed food products, which were positive for AFB1, 49% (22 samples) were found to have levels above the permissible limits of 5µg/kg.

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