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MYCOFLORA, MYCOTOXINS, BACTERIOLOGICAL ANALYSIS AND MOLECULAR ASSAY OF SOME BACTERIAL SPECIES FROM COFFEE BEANS IN SAUDI ARABIA

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اسفر التحليل الفطرى لبعض بذور البن بالمملكة العربية السعودية على مدى واسع من التلوث الفطرى لعدد ٣١ عينة تم تجميعها من سوبرماركت مختلفة بالرياض. تم عزل وتعريف ٤٣ نوعا من الفطريات تنتمى الى١٦ جنسا وعلى ٢٤ نوعا تنتمى الى ١٨ جنساعلى الوسطين الغذائيين جلوكوز وسليولوز اجار عند درجة حرارة ٢٥ درجة مئوية وذلك بطريقة عزل الفطريات بوضع البذور على سطح الوسط الغذائى. كانت الفطريات الشائعة فطرة الأسبرجلس نيجر يليها فطرة اسبرجلس فلافس بينما كانت فطرة بنسيليام اوكساليكم وسطية الانتشار، بينما تم عزل وتعريف ١٦ نوعا تنتمى الى ١٢ جنسا وعشرة انواع تنتمى الى ٨ اجناس على نفس الوسطيين الغذائيين بطريقة عمل تخفيفات مختلفة من غسل البذور وذلك عند درجة الحرارة درجة ٢٥ مئوية. وكانت فطرة اسبرجلس نيجرهى السائدة بينما كانت فطرة اسبرجلس فلافس وفطرة بنسيليام فنكيولوزم وسطية الانتشار. كانت فطرة اسبرجلس نيجر ايضاالاكثرشيوعا بينما كانت جنس ايروتيام بانواعه الثلاثة وسطية الانتشارعلى الوسط الغذائى ٢٠% سكروز تشابكس اجار عند درجة حرارة ٢٥ درجة مئوية. كذللك تم عزل ٥ انواع من الفطريات المحبة او المتحملة للحرارة على الوسط الغذائى مستخلص الخميرة والنشا وكانت تنتمى الى ٤ اجناس عند درجة الحرارة ٤٥ درجة مئوية، وكانت اكثرها شيوعا فطرة الاسبرجلس فيوميجاتس وتتبعها فطرةاسبرجيلس نيجر، بينما كانت الفطريات المحبة للحراة اقل شيوعا او نادرة. كما اسفرت النتائج بالتحليل الكروماتوجرافى بالاستخلاص بالكلوروفورم لعدد٣١ عينة من بذور البن عن خلو ٢٠ عينة تماما من السموم الفطرية بينما اظهرت ١١ عينة عن تلوثها بسموم الافلاتوكسين ب١ وب٢ وج١ وج٢ بتركيز يتراوح بين ١١ ـ ٦٠٠ ميكرون/جرام كذلك وجد ت ٦ عينات ملوثة بسموم بستريجماتوسستين بتركيز بين ٦٠ ـ ٦٠٠ ميكرون/جرام وبفحص الصفات الافرازية للسموم الفطرية لـ ٢٥ عزلة فطرية وجد ان ١٧ منهم انتجت سموم افلاتوكسين ب١ بتركز ٤٥٠ ميكرون/جرام واكراتوكسين ابتركيز ٦٠٠ ميكرون/جرام و سم اكراتوكسين ب بتركيز ٤٠٠ ميكرون/جرام واسترجماتوسستين بتركيز ٢٨٠ ميكرون/جرام وهى من انواع الاسبرجيلس، بينما انتجت ٣ عزلات من البنسيليام حامض البنيسيليك بتركيز يتراوح بين ٢٤٠ ـ ٧٢٠ ميكرون/جرام وعزلة واحدة من فطرة ترايكوديرما انتجت سم ترايكودرمين. كما اوضحت

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التحاليل البكتريولوجية لبذور البن عن ظهور بكتيرة باسيلس سيرياس فى ٦ عينات بن بتركيز ١٠×٢ مستعمرة بكتيرية وبكتيرة اشيريشيا كولاى فى ٢ بتركيز ١٠×١٤٠ و ١٠×٨٩ وظهرت بكتيرة فيكال كولاى فورم فى عينة واحدة بتركيز١٠×٤ بينما ظهرت بكتيرة استافيلوكوكاس فى ٢٩ عينة وكان اعلى تركيز ١٠×٥٥ ^{٣} ، كما اظهرت النتائج خلو العينات جميعها من بكتيريا سالمونيلا. كذلك تم التحليل البكتيرى لعينات بذور البن باستخدام البسى آر البيولوجيا الجزيئية ووجد ان جميع العينات سالبة لبكتيريا يريزينا انتروكوليتيكا وكامبيلوباكتر وليستيريا منوسيتوجين وكانت النتائج مطابقة لنتائج بكتيرة باسيلس سيرياس و بكتيرة سالمونيلا.

The mycoflora analysis of some coffee beans in Saudi Arabia showed a wide range of fungal contamination in 31 samples collected from different markets in El-Riyadh. Thirty four species belonging to 16 genera and 28 species belonging to 18 genera were isolated from coffee beans on glucose and cellulose Czapek's agar medium at 25°C from seed-plate method. Aspergillus niger and A. flavus were the most prevalent species, but Penicilliun oxalicum was isolated in moderate occurrence, while 12 genera comprised 16 species and 8 genera comprised 10 species were isolated on the same types of media at 25°C from seed suspension method. A. niger was the most common species, while A. flavus and P. funiculosum were isolated in moderate occurrence. A. niger, also was the most prevalent on 20% sucrose-Czapek's agar medium at 25°C, but the genus Eurotium (3 spp) appeared in moderate occurrence. Five fungal species belonging to four genera were isolated on starch yeast extract agar medium at 45°C. A. fumigatus and A.niger were the most prevalent thermo tolerant species, while three species of thermophilic fungi were of low or rare incidence.

Thin layer chromatographic analysis of chloroform extracts of 31 coffee beans samples revealed that 20 samples were free from mycotoxins, while 11 samples were contaminate with aflatoxins B^1, B^2, G^1 and G^2 of concentrations ranged from 110-600 ug/kg, but 6 samples were contaminate with sterigmatocystin ranged from 60-600 ug/kg. Screening of the characteristic mycotoxins of 25 fungal isolates revealed that 17 of them produced, aflatoxin B^1 at 450 ug/kg, ochratoxin A at 600 ug/kg, ochratoxin B at 400 ug/kg, and sterigmatocystin 280 ug/kg from Aspergillus species, while three isolates of Penicillium produced penicillic acid (ranged from 720-240 ug/kg) and one isolate of Trichoderma produced Trichodermine at 360 ug/kg. The bacteriological analysis of the coffee bean samples indicated that Bacillus cereus was detected in six samples at levels (2x10 cfu/g), E. coli in two samples (14x10 and 89x10 cfu/g), Feacal coliform was detected in one sample only,

while Staphylococcus appeared in 29 samples (55x103 cfu/g). All samples were free from Salmonella.

PCR assay for detection of some bacterial species revealed that all samples were negative for Yersinia enterocolitica, Campylobacter and Listeria monocytogenes, while the results of B. cereus and Salmonella were similar to the results obtained using cultural method.

INTRODUCTION

Coffee Arabica originated in Ethiopia. It is believed to have been introduced into Arabia prior to the 15th century[1]. Today, nearly 90% of the world's coffee come from this species[2]. Yemen Mocha coffee is considered to be one of the world's greatest, uniquely delicious coffees[3]. Dried seeds "beans" are Roasted ground and brewed to make one of the two most important beverages in the western world. Coffee is widely used as a flavoring, as in ice cream, pastries, candies and liqueurs. Source of caffeine, dried ripe seeds of coffee are used as a stimulant, nervine, and diuretic, acting on central nervous system, kidneys, heart, and muscles[1]. Scudamore *et al.*[4] Demonstrated the influences of roasted coffee bean aroma on rat brain which changes the mRNA and protein expressed, or stress relaxation activities of the coffee bean aroma. Many fungi attract Arabica coffee plants, among them, *Fusarium moniliform*, *F. solani*, *F.oxysporum*, and others[1]. *Aspergillus restrictus* was isolated from coffee[5]. *A. niger* infecting Vietnamese coffee beans and Vietnamese green coffee beans were more severely infected with fungi than the levels reported for beans from other part of the world[6]. Several authors studied the fungal flora of coffee beans[7-14]. In Egypt, Abdel-Hafez and El-Maghraby[15] studied the mycoflora of roasted coffee, and also in Yemen[3,16&17].

Although the natural occurrence of mycotoxins on coffee beans were studied as described, previously[6,9&18-22]. In Egypt[23], while in Yemen[3] from coffee beans and[7] from dried fruits.

Bacterial isolation from beans, also were studied, for example, from coffee cherries[11]; form fermenting coffee[24] and from fermenting cacao[25&26].

Several authors studied molecular identification and PCR to identification of microorganisms[27-31].

The aim of this study is to survey of mycobiota and mycotoxins contaminated coffee beans collected from different locations in Al-Riyadh, Saudi Arabia and testing the ability of certain fungal isolates to produce mycotoxins and to determine the bacteriological status of coffee bean samples and PCR assay for detection of these bacteria.

MATERIAL AND METHODS

Collection of coffee samples

Thirty-one samples of coffee beans were randomly collected from different locations in Al-Riyadh, Saudi Arabia which were cultivated in Khawlan in Yemen and Harare in Ethiopia (Table 1). Each sample was put in a sterile polyethylene bag, sealed and put in another bag which was also sealed. Storage placed.

Isolation and identification of fungi

Fungi were detected using two methods. The first is seed-plate method as described by[32], in which 4 seeds were placed on the surface of sterile media. Five plates were used for each sample and each medium; the plates were incubated for 5-7 days at 25°C. The second method is the dilution plate method as described previously[33] was used for isolation of fungi. Five gram seeds of each sample were placed in a 500 ml sterilized distilled water in Erlenmeyer flask, and shaken for 15 min. One ml of seed suspension was placed into each Petri dish, 12-15 ml of melted and cooled medium were poured. Five plates were used for each sample, and for each medium.

Glucophilic fungi were cultured on glucose-Czapek's agar medium in which glucose (10 gm/l) replaced sucrose.

Cellulose-Czapek's agar in which glucose was replaced by cellulose powdered (20 gm/l).

Osmophilic and osmotolerant fungi were allowed to grow on sucrose-Czapek's agar which contained 200 gm/l sucrose instead of glucose.

Thermophilic and thermotolerant fungi were cultured on starch yeast agar (YpSs) which contained gm/l: Soluble starch, 20; yeast extract, 4; KH_2PO_4, 1; $Mgso_4.7H_2O$, 0.5 and agar, 15 gm. All types of media were supplemented with chloramphenicol (20 ug/ml) and Rose Bengal (30ppm) as bacteriostatic agent. Pure cultures of fungi were kept in slant agar tubes which contained the appropriate medium provided with 0.5 g chloramphenicol[34].

Identification: Purified fungal isolates were identified morphologically (based on macroscopic and microscopic characteristics), whenever possible, in the original Petri-dish culture. When this was not possible, fungi were subculture and stored for later identification according to Simmons[11], Booth[35], Domsch *et al.*[36], Ellis[37], Ellis[38], Moubasher[39], Onion *et al.*[40], Pitt[41], Pitt[42], Raper and Fennel[43], Raper and Thom[44], Rifai[45] and Samson[46].

Determination of mycotoxins

Extraction of coffee bean samples for natural occurrence of mycotoxins

The samples were stored at 22°C for 1, 2, 3, and 4 months then extracted for the presence of aflatoxins B_1, B_2, G_1, G_2, ochratoxin A and sterigmatocystin. During these

periods the rate of fungal growth was determined visually as described previously[47].

Twenty gram of each sample were defatted by extraction with cyclohexane for 10 h using a Soxhlet-type extractor. The defatted residue was extracted for another 10 h with chloroform. The chloroform extract was dried over anhydrous sodium sulphate, filtered and then evaporated under vacuum to near dryness. The residue was diluted with chloroform to one ml.

Detection of mycotoxins

Thin layer chromatographic technique of the clean extract was done on percolated silia gel Plate type 60 F254 (Merck) for the presence of mycotoxins according to standard procedures described[46,48&49].

Verification of different recorded mycotoxins

Simple configuration methods of recorded mycotoxins on precoated silica gel plates were described[46&50-52]. The TLC plates commonly used are normal phase silica gel plates. For some acidic metabolites like cyclopiazonic acid, citrinin, luteoskyrin etc., it can be useful to impregnate the plate with oxalic acid. This is simply done by dipping the plate in an 8% solution of oxalic acid in water or methanol followed by air drying.

TLC-conditions[46]

After application the TLC-plate, any suitable TLC-procedure can be performed. It was found the following solvents (saturated conditions) very useful:

TEF: Toluene / Ethyl Acetate / Formic acid (90%) 5:4:1

CAB: Chloroform / Acetone / Iso propanol 85:15:20

CM: Chloroform / Methanol 97:3

After elution and air drying in a dark fume hood, the TLC-plates are examined in visible light (VIS), long wave UV-light (UV-366) and short wave UV-light (UV-254) some metabolites are treated with 1/2 minute in UV-254 followed by UV-366.

The following spray reagents are useful for visualizing and verification of secondary metabolites:

Spray 1: 0.5% p-anisaldehyde in ethanol / acetic acid / conc. sulphuric acid 17: 2:1 (most metabolites).

Spray 2: 50% sulphuric acid in water (e.g. aflatoxins B_1 & B_2; verruculogen; viridicatins; cyclopiazonic; streigmatocystin; T-2 toxin.

Spray 3: $FeCl_3$ in butanol and heating for 5 min. at 130°C (e.g. Aspergillic acid; kojic acid; penicillic acid; citrinin; verrucologen.

Spray 4: 20% $AlCl_3$ in 60% ethanol and heating for 5 min. at 130°C (e. g. penitrem A; trichothecenes B; sterigmatocystin; gliotoxin; T-2 toxin.

Spray 5: NH_3 vapour in 1-3 min (mycophenolic acid; xanhomegnin; viomellien, penicillic acid; ochratoxin A; kojic acid; citrinin; patulin).

Extraction of mycotoxins from fungal isolates

Culture of selective 25 fungal isolates collected from the current study was examined. The tested samples were represented by seven species of *Aspergillus* as the following: *A. flavus* (5 isolates), *A. melleus* (2 isolates), *A. ochraceus* (one isolate), *A. sclerotioniger* (one isolate), A. terreus (one isolate), *A. ustus* (2 isolates), and *A. versicolor* (one isolate); one species of *Paecillomyces variotii* (2 isolates); five species of *Penicillium, P. funiculosum* (2 isolates), *P. glabrum* (one isolate), *P. islandicum* (one isolate), *P. oxalicum* (one isolate), *P. chrysogenum* (3 isolates) and two isolates of *Trichoderma harizianum* (Table 8). Inocula were prepared from 7-days old culture of each isolates on PDA slope as spore suspensions in 0.2% aqueous tween 80 (v/v). Isolates were inoculated into 250 ml Erlenmeyer flasks each containing 50 ml Capek's liquid medium supplemented with 0.2% yeast extract and 1.0 peptone and incubated at 28°C for 10 days as static culture (PYCZ).

Extraction of fungal toxins

After incubation, the control of each flask (medium + mycelium) was homogenized for 5 min in a high-speed blender with 100 ml chloroform. The extract procedure was repeated three times. The chloroform extracts were combined, washed, dried, filtered and concentrated to near dryness, cleaned and mycotoxins are detected as previously described[46&53].

Bacterial analysis

A total of 31 coffee beans samples illustrated in Table (1) were analyzed for the Total Plate Count[54], Total coliform count[55], Faecal coliform count according to NMKL[56], *Staphylococcus c*ount according to NMKL[57], *Bacillus cereus* count according to NMKL[58], *Salmonella* count according to NMKL[59] and Total fungal count according to NMKL[56]. The method is summarized as follows: 5 grams of the sample were mixed with 45 ml of the relevant diluents from which tenfold serial dilution were made down to the expected contamination level. Three ml from each dilution were inoculated each in one sterile Petri dishes in which relevant specific media was poured and after solidification the 3 plates were incubated at the relevant temperature for the relevant period of time.

PCR for detection of bacterial species (Table 2)

DNA extraction buffer: according to Samson *et al*[60]

All tested samples were enriched in Buffered Peptone Water (BPW) for 24 hrs at 37°C before subjected to DNA extraction. DNA extraction was performed using Prepman Ultra Sample Preparation Reagent, Applied Biosystem, USA, according to the user manual attached to the Kit as follows: One ml from the enriched BPW was transferred to 2 ml microcentrifuge tube and spinned for

Table 1: Number, source and name of samples.

No. Samples	Source	Name
1	Yemen	Yemeni
2	Yemen	Yemeni
3	Yemen	Yemeni
4	Yemen	Yemeni
5	Yemen	Yemeni
6	Yemen	Yemeni
7	Yemen	Yemeni
8	Ethiopia	Harare
9	Yemen	Yaphai
10	Yemen	Harare
11	Yemen	Khawlani
12	Ethiopia	Harare
13	Ethiopia	Harare
14	Yemen	Yemeni
15	Yemen	Yemeni
16	Yemen	Yemeni
17	Yemen	Yemeni
18	Yemen	Yemeni
19	Yemen	Yemeni
20	Yemen	Yaphai
21	Yemen	Khawlani
22	Yemen	Yemeni
23	Ethiopia	Harare
24	Ethiopia	Harare
25	Ethiopia	Harare
26	Yemen	Yemeni
27	Yemen	Yemeni
28	Yemen	Yemeni
29	Yemen	Yemeni
30	Yemen	Yemeni
31	Yemen	Yemeni

Table 2: Primer and probe sequences of bacteria under investigation.

Item	Nucleotides, sequence
Listeria monocytogenes Forward primer sequence:	TGC AAG TCC TAA GAC GCCA
Listeria monocytogenes Reverse primer sequence:	CAC TGC ATC TCC GTG GTA TAC TAA
Listeria monocytogens Probe	6-FAM - CGA TTT CAT CCG CGT GTT TCT TTT CG - TAMRA
Bacillus cereus Forward primer sequence	GTG TTT GAC CAA GGT GGA CAA
Bacillus cereus Reverse primer sequence	TTA CTC CAT AGA GCA CCC TTG GA
Bacillus cereus Probe	6-FAM - CCA AAA CCA GTT GCC AGT GCA TTGG - TAMRA
Campylobacter jeujeni Forward	TTG GTA TGG CTA TAG GAA CTC TTA TAG CT
Campylobacter jeujeni Reverse	CAC ACC TGA AGT ATG AAG TGG TCT AAG T
Compylobacter jeujeni Probe	6-FAM - TGG CAT ATC CTA ATT TAA ATT ATT TAC CAG GAC - TAMRA
Yersinia enterocolitica Forward	AAT GCT GTC TTC ATT TGG AGC
Yersinia enterocolitica Reverse	ATC CCA ATC ACT ACT GAC TTC
Yersinia enterocolitica Probe	6-FAM - CAA GCA AGC TTG TGA TCC TCC G - TAMRA
Salmonella typhimurium Forward	TCG TCA TTC CAT TAC CTA CC
Salmonella typhimurium Reverse	AAA CGT TGA AAA ACT GAG GA
Salmonella typhimurium Prpbe	6-FAM - TCT GGT TGA TTT CCT GAT CGC A - TAMRA

3 min at 16000 rpm. The resulted supernatant was discarded and the obtained pellet was eluting using 200 ul of Perpman Ultra Sample Preparation Reagent. The tube then was incubated in a boiling water bath for 10 min with frequent mixing. After reaching room temperature, the tube was centrifuged at 16000 rpm for 3 min then 50 ul from the supernatant was transferred to a new labeled micro centrifuge tube.

TaqMan RT-PCR assay for detection of bacterial species

PCR was performed in reaction mixture with a total volume of 25 ul containing 1 ul of extracted DNA, 0.5 mM of each primers, 0.2 mM of fluorogenic probe, and TaqMan Universal Master Mix (Applied Biosystems, USA). The Master Mix contained Amp Erase uracil-N-glycosylase (UNG), deoxynucleoside triphosphate with dUTPs, 6-carboxyrhodamine as an internal passive fluorogenic reference, and an optimized buffer component. Amplification and detection were carried out in optical- grade 96- well plates in an ABI Prism 7000 sequence detection system (Applied Biosystems) with an initial step of 50°C for 2 min, which is required optimal Amp Erase UNG enzyme activity, and then at 95°C for 10 min, to activate the AmpliTaq Gold DNA polymerase and to deactivate the AmpEease UNG enzyme, followed by 40 cycles of 95°C for 15 s and 60°C for 1min. The reaction conditions for amplification and the parameters for fluorescence data collection were programmed into a Dell laptop linked directly to the ABI Prism 7000 sequence detection system by using the SDS 1.6 application software, according to the manufacture's instructions. After real-time data acquisition, the threshold, which was defined as being 10-foldfiger than the baseline, was determined, and the cycle threshold (CT) value was manually set so that it intersected the amplification curves in the linear region of the semi log plot.

RESULTS AND DISCUSSION

Mesophilic fungi

Fungi recovered from seed-plate method on glucose and cellulose-Czapek's agar media at 25°C: The total viable fungal propagules ranged from 942-891 colonies / 1240 seeds of all coffee bean samples tested on both glucose and cellulose Czapek's agar media. Thirty-four species belonging to 16 genera and 28 species belonging to18 genera were isolated on both glucose and cellulose-Czapek's agar media at 25°C (Table 3). All of these fungi were previously recovered from coffee beans[3,16&17], also from different places of the world[1,6,9,11,13,14,61&62].

Aspergillus was the most prevalent genus, recorded in 100% of the samples on the two isolation media used. It was represented by 10 species on glucose and 5 species on cellulose Czapek's agar media. *A. niger* was the most common *Aspergillus* species, appeared in 100% of the samples, contributing (61.4 and 61.7% of total fungi) on the

Table 3: Counts (of colonies / 1240 seeds) and Incidences (out of 31 samples) of mesophilic fungi isolated from coffee seeds on glucose and cellulose-Czapek's agar.

Fungal species	Glucose-Czapek's agar				Cellulose-Czapek's agar			
	Total count	% Total count	Incidence	% Incidence	Total count	% Total count	Incidence	% Incidence
Aspergillus	675	71.8	31	100	636	71.4	31	100
Aspergillus flavus Link	36	3.8	16	51.6	45	5.1	16	51.6
Aspergillus níger van Tieghem	578	61.4	31	100	550	61.7	31	100
Aspergillus ochraceus Wilhelm	34	3.6	5	16.1	33	3.7	4	12.9
Aspergillus ustus Thom & church	15	1.6	1	3.2	0	0.0	0	0.0
Aspergillus clavatus Desm	1	0.1	1	3.2	0	0.0	0	0.0
Aspergillus fumigatus Fresenius	2	0.2	1	3.2	2	0.2	2	6.5
Aspergillus sclerotioniger	7	0.7	4	12.9	0	0.0	0	0.0
Aspergillus candidus Link	1	0.1	1	3.2	0	0.0	0	0.0
Aspergillus tamarii Kita	1	0.1	1	3.2	0	0.0	0	0.0
Aspergillus terreus Thom	2	0.2	1	3.2	6	0.7	5	16.1
Acremonium	15	1.6	5	16.1	5	0.5	3	9.7
Acremonium roseoium Link	9	1	2	6.5	3	0.3	1	3.2
Acremonium strictum Gams	6	0.6	3	9.7	2	0.2	2	6.5
Alternaria alternata (Fr.)Keissler	10	1	6	19.4	16	1.8	5	16.1
Cephaliophora tropica Thaxter	1	0.1	1	3.2	0	0.0	0	0.0
Cheatomium globosum Kunze	0	0.0	0	0.0	1	0.1	1	3.2
Circinella muscae	0	0.0	0	0.0	1	0.1	1	3.2
*Cladosporium cladosporioides*Vries	30	3.2	16	51.6	45	5.1	16	51.6
Cochliobolus spicifer Nelson	0	0.0	0	0.0	2	0.2	1	3.2
Curvularria lunatus Nelson	1	0.1	1	3.2	0	0.0	0	0.0
Dark Sterile hyphae	7	0.7	3	9.7	0	0.0	0	0.0
Epicoccum nigrum Link	4	0.4	2	6.5	4	0.5	2	6.5
Emericella nídulans (E.) Vuillemin	3	0.3	2	06.5	3	0.3	3	9.7
Eurotium repens De Bary	1	0.1	1	3.2	0	0.0	0	0.0

Table 3: Continued

Fungal species	Glucose-Czapek's agar				Cellulose-Czapek's agar			
	Total count	% Total count	Incidence	% Incidence	Total count	% Total count	Incidence	% Incidence
Fusarium solani (Mart) Sacc.	0	0.0	0	0.0	2	0.2	2	6.5
Mucor hiemalis Wehmer	8	0.9	5	16.1	10	1.1	10	32.3
Paecilomyces variotii Bainier	10	1	4	12.9	8	0.9	2	6.5
Penicillium	64	6.8	24	77.4	50	5.6	22	71
Penicillium aurantiogriseum Diercky	0	0.0	0	0.0	1	0.1	1	3.2
Penicillium chrysogenum Thom	0	0.0	0	0.0	5	0.6	3	9.7
Penicillium citrinum Thom	1	0.1	1	3.2	0	0.0	0	0.0
Penicillium funiculosum Thom	12	1.3	7	22.6	10	1.1	7	22.6
Penicillium glabrum We. Westling	1	0.1	1	3.2	0	0.0	0	0.0
Penicillium islandicum	6	0.6	3	9.8	19	2.1	4	12.9
Penicillium purpurogenum	1	0.1	1	3.2	0	0.0	0	0.0
Penicillium oxalicum Thom	43	4.6	11	35.5	15	1.7	7	22.6
Phoma herbarum Westendrop	0	0.0	0	0.0	2	0.2	2	6.5
Rhizopus	38	4	16	51.6	37	4.2	14	45.2
Rhizopus oryzae	34	3.6	13	41.9	37	4.2	14	45.2
Rhizopus stolonifer (Ef.) Lindt	4	0.4	3	9.7	0	0.0	0	0.0
Scopulariopsis	0	0.0	0	0.0	2	0.2	2	6.4
Scopulariopsis brevicaulis (Sa) Bain	0	0.0	0	0.0	1	0.1	1	3.2
Scopulariopsis candida	0	0.0	0	0.0	1	0.1	1	3.2
Stemphilum botryosum Wallorth	6	0.6	2	6.5	1	0.1	1	3.2
Trichoderma harzianum	46	4.9	4	12.9	40	4.5	2	6.5
Ulocladium chartarum Preuss	3	0.3	3	9.7	0	0.0	0	0.0
White sterile mycelium	5	0.5	5	16.1	21	2.4	9	29
Yeast Sp.	13	1.4	5	16.1	5	0.6	4	12.9
Growss Total Count	942	-	-	-	891	-	-	-
No. of genera	16	-	-	-	18	-	-	-
No. of species	34	-	-	-	28	-	-	-

two types of media. *A. flavus* was the second common species which gives high incidence, occurring in 51.6% of the samples comprising (3.8 and 5.1% of total fungi) on both glucose and cellulose Czapek's agar media respectively. It was previously reported that *Aspergillus* was the most prevalent genus isolated from coffee beans and *A. flavus, A. fumigatus* and *A. niger* were the most dominant on coffee samples cultured on different medium types[3]. However, data previously obtained[16&17] induced the dominant of *Aspergillus* in 100% of the samples of coffee fruits used and *A. niger, A. flavus* were the most common species. In this study, the remaining other *Aspergillu*s species were isolated with low or rare incidence (Table 3). The preceding *Aspergillus* species were isolated previously, but with variable densities and frequencies from powdered coffee samples[15], from coffee beans, in Yemen[3,16&17] and from different part of the world[6,9,11,13,14,61&62].

Penicillium came second in incidence. It was isolated in high frequency on the two isolation media used. It was recovered from 77.4 and 71% of the samples, giving rise to 6.8 and 5.6% of total fungi on glucose and cellulose- Czapek's agar, respectively. It was represented by 6 and 5 species on glucose and cellulose-Czapek's agar media respectively, of which *P. oxalicum* was isolated in moderate incidence (35.5% of the samples), comprising 4.6% of total fungi on glucose-Czapek's agar medium, while appeared in low incidence on cellulose-Czapek's agar medium. The remaining other *Penicillium* species were recovered in low or rare incidence (Table 3). Also, previous results[16&17], indicated that *Penicillium* was one of the most fungal genera recorded on coffee fruits in Yemen, while[3], reported that *Penicillium* appeared in low and rare incidences on coffee fruits or beans. *P. oxalicum* was recovered, but with variable densities and frequencies from bean seeds gathered from Upper Egypt[63&64].

Cladosporium cladosporioides occupied the third place with regard to the total count. It occurred in 51.6% of the samples on the two types of media used. Abadel-Hafez and El-Maghraby[15] studied the fungal flora of roasted coffee, and recoded that *C. herbarum* was one of the most common species. *Rhizopus* (2 species) was isolated with high incidence on glucose-Czapek's agar medium only. *R. oryzae* was encountered in moderate incidence on the two isolation media used. *R. stolonifer* occurred in low to moderate frequency on coffee samples[3]. The remaining other genera and species were isolated in low or rare frequencies (Table 3).

Fungi recovered from spore-suspension of coffee seed on glucose and cellulose Czapek's agar media at 25°C: Twelve genera comprised 16 species and 8 genera comprised10 species were recovered on both glucose and cellulose Czapek's agar medium respectively, at 25°C.

Aspergillus was the most common genus on the two media, comprised 87% and 71% on glucose and cellulose Czapek's agar media respectively. It was represented by 4 species and 2 species on both glucose and cellulose-Czapek's agar media. *A. niger* was the most dominant species on the two media, it was estimated in 87.1% and 71% of the samples (Table 4). *A. flavus* appeared in moderate occurrence, constituting 22.6 and 38.7% of total samples on glucose and cellulose-Czapek's media respectively. The remaining *Aspergillus* species on glucose-Czapek's medium were recovered in low or rare incidences. *Penicillium* appeared in moderate occurrence (22.6% of the samples) on glucose-Czapek's medium and in low incidence (9.7% of samples), on cellulose-Czapek's medium. It was represented by *P. funiculosum* (6.5 and 9.7%) on the two media and *P. oxalicum* (16.1%) on glucose-Capek's agar medium only. The preceding genera and species were previously isolated, but with variable densities and frequencies from different types of seeds[52&63-69]. From coffee beans[3,16&17], also from different places of the world[1,6,9,11,13,14,61&62].

The remaining other fungal species appeared in low or rare occurrence (Table 4).

Osmophilic fungi: Fungi recovered from seed-plate method on sucrose-Czapek's agar medium at 25°C: Ten species of fungi belonging to 6 genera were isolated from coffee seeds on osmophilic 20% sucrose-Czapek's agar medium at 25°C. The average total count of fungi in all samples tested was 491 colonies / 620 seeds. All of these fungi were previously recovered from different beans, peas and other types of seeds[63,64&67].

Aspergillus was the most common genus, recovered in 96.8% of the samples, and it was represented by 3 species. *A. niger* was isolated in high occurrence contributing to 96.8% of the samples; the remaining 2 species appeared in rare frequencies (Table 5). *Eurotium* was recorded in moderate incidence, comprised 41.9% of total samples. It was represented by 3 species *E. amstelodami* (32.3), *E. chevallieri* (35.5) and *E. repens* (41.9%), were recovered in moderate frequencies also, Al-Kolaibe[3] reported that these three osmophilic fungal species belonging to the genus *Eurotium* appeared only on 40% sucrose-Czapek's, found in 52% of samples sharing with 33.0% of total fungi, while Alghalibi and Shater[7], recorded *E. amstelodami* isolated from dried fruit samples was one of the common fungi on 40% sucrose-Czapek's medium. Also, the genus *Eurotium* was isolated with high occurrence on 40% sucrose-Czapek's agar[70]. The remaining other species were recovered in rare occurrences Table (5).

Thermophilic and thermotolerant fungi recovered from spore-plate method on starch yeast extract medium at 45°C: Five fungal species belonging to 4 genera were isolated from coffee beans on yeast starch agar medium at 45°C (Table 6).

Table 4: Counts (of colonies / g) and Incidences (out of 31 samples) of spore suspension of mesophilic fungi isolated from coffee beans on glucose and cellulose agar media at 25°C.

Fungal species	Glucose-Czapek's agar				Cellulose-Czapek's agar			
	Total Count	% Total Count	Incidence	% Incidence	Total Count	% Total Count	Incidence	% Incidence
Acremonium strictum	36	0.5	2	6.5	20	0.2	1	3.2
Alternaria alternata	32	0.4	3	9.7	0	0.0	0	0.0
Aspergillus	7043	94.6	27	87.1	9636	85.5	22	71
Aspergillus flavus	403	5.4	7	22.6	2680	23.8	12	38.7
Aspergillus Níger	6544	87.9	27	87.1	6956	61.8	22	71
Aspergillus sclerotioniger	48	0.6	2	6.5	0	0.0	0	0.0
Aspergillus terreus	48	0.6	3	9.7	0	0.0	0	0.0
Circinella muscae	8	0.1	1	3.2	0	0.0	0	0.0
Cladosporium cladosporioides	104	1.4	4	12.9	16	0.1	1	3.2
Epicoccum nigrum	24	0.3	2	6.5	0	0.0	0	0.0
Mucor hiemalis	24	0.3	3	9.7	16	0.1	2	6.5
Paecilomyces variotii	0.0	0.0	0	0.0	180	1.6	1	3.2
Penicillium	93	1.3	7	22.6	48	0.4	3	9.7
Penicillium funiculosum	29	0.4	2	6.5	48	0.4	3	9.7
Penicillium oxalicum	64	0.9	5	16.1	0	0.0	0	0.0
Rhizopus oryzae	16	0.2	2	6.5	0	0.0	0	0.0
Scopulariopsis brevicaulis	16	0.2	2	6.5	0	0.0	0	0.0
Trihcoderma	40	0.5	2	6.5	1340	11.9	3	9.7
Trichoderma harzianum	40	0.5	2	6.5	1020	9.1	2	6.5
Trichoderma koningii	0.0	0.0	0.0	0.0	320	2.8	1	3.2
Ulocladium botrytis	8	0.1	1	3.2	8	0.1	4	12.9
Yeast Sp.	16	0.2	2	6.5	0	0.0	0	0.0
Growss Total Count	7444	-	-	-	11264	-	-	-
No. of genera	12	-	-	-	8	-	-	-

Table 5: Counts (colonies / 620 seeds) of osmophilic fungi isolated from coffee seeds on 20% sucrose Czapek's agar at 25°C.

Fungal species	Total Count	% Total Count	Incidence	% Incidence
Aspergillus	376	76.6	30	96.8
Aspergillus flavus	4	0.8	2	6.5
Aspergillus Níger	367	74.7	30	96.8
Aspergillus sclerotioniger	5	1	1	3.2
Cladosporium cladospoiroides	2	0.4	1	3.2
Emericella nidulans	2	0.4	1	3.2
Eurotium	105	21.4	13	41.9
Eurotium amestlodami	28	5.7	10	32.3
Eurotium chevalieri	30	6.1	11	35.5
Eurotium repens	47	9.6	13	41.9
Mucor hiemalis	4	0.8	2	6.5
Rhizopus oryzae	2	0.4	1	3.2
Growss Total Count	491	-	-	-

Table 6: Counts (colonies / 620 seeds) of thermotolerent and thermophilic fungi isolated from coffee seeds on starch yeast extract agar (YpSs) at 45°C.

Fungal species	Total Count	% Total Count	Incidence	% Incidence
Aspergillus	160	86	20	64.5
Aspergillus fumigatus Fresenius	63	33.9	15	48.4
Aspergillus Níge Van Tieghem	97	52.2	20	64.5
Rhizomucor pusillus Schipper	15	8.1	6	19.4
Talaromomyces dupontii (Thom) Beniamin	1	0.5	1	3.2
Thermomyces lanuginosus Tsiklinsky	10	5.4	5	16.1
Growss Total Count	186	-	-	-

Aspergillus was the most prevalent genus, appeared in high incidence matching 86% of total fungi and 64.5% of total samples. It was represented by two species, the thermotolerent *A. fumigatus* (48.4% of the samples), and *A. niger* (64.5% of the samples). However, the remaining three fungal species were of true thermophilic fungi, *Rhizomucor pusillus*, *Talaromyces dupontii* and *Thermomyces lanuginosus*, were recovered in low or rare incidences Table (6). These results agreed with the results of Al-Kolaibe[3] who observed that *Aspergillus* was the dominant genus of the three genera isolated at 45°C; *A. fumigatus* and *A. niger* were the major representatives of the genus.

Natural occurrence of mycotoxins

The results of thin layer chromatographic analysis of 31 coffee bean samples were randomly collected from different markets in El-Riyadh, Saudi Arabia which cultivated in Yemen (Khawlan and Wild plants) and Ethiopia.

Naturally occurrening of mycotoxins in coffee bean seed samples

Twenty samples tested of coffee beans were free from mycotoxins, while eleven samples tested of coffee seeds were contaminated with aflatoxin B_1 No. (14, 6, 5, 28, 29, 27, 30 and 31) ranged from 110 ug - 600 ug/kg from Yemen, and only one sample from Khawlan contaminate with aflatoxin B_1 120 ug/kg, also, aflatoxin B_2 No. (28, 27, 29, 30 and 31) ranged from 360-600 ug/kg from Yemen, aflatoxins G_1 and G_2 were 600 ug/kg, one sample No. (25) from Harari, Ethiopia contaminate with aflatoxin B_1 at 110 ug/kg. Also, sterigmatocystin samples No. (15, 27, 28, 29, 30 and 31) ranged from 60-600 ug/kg. Twenty samples were free from mycotoxins from Yemen, Wild plants, and Khawlan in Yemen, and one sample from Harare, Ethiopia (Table 7). Nakajima *et al.*[71] recorded that 32% of coffee bean samples (47 samples) were contaminated with aflatoxins. Levi[72] found that aflatoxin B_1 contaminated 2 green coffee bean samples out of 201 tested at a level of 3-12 ug/kg. While Tsubouchi *et al.*[61] observed that 4 samples out of 22 tested were contaminated with 9.9-46 ug/kg of ochratoxin A. El-Maraghy[69] reported the presence of aflatoxins B_1, B_2, G_1 and G_2 in the extract of one variety of chick-pea out of five varieties tested.

Sterigmatocystin was isolated from 5 coffee seeds samples in this study However, it was previously[3] reported that sterigmtocystin contamination in green coffee beans are very rare. Purchase and Pretorius[73] found that one sample of green coffee beans out of 2 was contaminated with sterigmatocytosin at concentration 1.14 ppm.

Table 7: Naturally occurring mycotoxins in coffee seeds.

Sample No.	Locality	Toxins detected	Quantity (ug/kg)
1	Yemen	--	--
2	Yemen	--	--
3	Yemen	--	---
4	Yemen	--	---
5	Yemen	Aflatoxin B1	240 µg
6	Yemen	Aflatoxin B1	120 ug
7	Yemen	-	-
8	Harari, Ethiopia	--	---
9	Wild Plants, Yemen	--	--
10	Harari, Ethiopia	--	---
11	Khawlan, Yemen	--	--
12	Harari, Ethiopia	--	--
13	Harari, Ethiopia	--	--
14	Yemen	Aflatoxin B1	110 µg
15	Yemen	Aflatoxin B1 Sterigmatocystin	600 µg 180 µg
16	Yemen	-	-
17	Yemen	--	--
18	Yemen	--	--
19	Yemen	--	--
20	Wild Plants, Yemen	--	--
21	Khawlan, Yemen	Aflatoxin B1	120 µg
22	Yemen	--	--
23	Harari, Ethiopia	--	--
24	Harari, Ethiopia	--	--
25	Harari, Ethiopia	Aflatoxin B1	110 µg
26	Yemen	--	---
27	Yemen	Aflatoxin B1 Aflatoxin B2 Sterigmatocystin	460 µg 460 µg 60 µg
28	Yemen	Aflatoxin B1 Aflatoxin B2 Strigmatocystin	360 µg 360 µg 180 µg
29	Yemen	Aflatoxin B1 Aflatoxin B2 Sterigmatocystin	360 µg 360 µg 60 µg

Table 7: Continued

Sample No.	Locality	Toxins detected	Quantity (ug/kg)
30	Yemen	Aflatoxin B1	600 µg
		Aflatoxin B2	600 µg
		Aflatoxin G1	600 µg
		Aflatoxin G2	600 µg
		Sterigmatocystin	250 µg
31	Yemen	Aflatoxin B1	600 µg
		Aflatoxin B2	600 µg
		Aflatoxin G1	600 µg
		Aflatoxin G2	600 µg
		Sterigmatocystein	120 µg

Mycotoxins produced by fungi isolated from coffee seeds

Twenty-five isolates were randomly selected from fungal cultures of coffee beans. They comprised 5 isolates attributed to *Aspergillus flavus* (number 11, 27, 12, 22) produced aflatoxins B_1 (Fig. 1) from 450 ug/kg and one isolate (No. 19) at 750 ug/kg, one isolate of *A. melleus* produced ochratoxine A (Fig. 2) at 600 ug/kg, two of *A. ochraceus* and *A.sclerotioniger* (No. 11 and 1) produced ochratoxins B (Fig. 2) at 400 ug/kg, one sample of *A. versicolor* (No. 25) produced sterigmatocystin (Fig. 1) at 280 ug/kg, two of *Penicillium funiculosum* and one of *P. islandicum* No. (1, 2 and 3) produced Penicillic acid (Fig. 3) ranged from 720-240 ug/kg and 480 ug/kg respectively, and one isolate of *Trichoderma harizianum* No. 17 produced Trichodermine (Fig. 1) at 360 ug/kg. On the other hand 8 isolates proved to be free from mycotoxins Table (8). Many reports have indicated that not all isolates of these species can produce aflatoxins. Abd-Alla *et al.*[23] and Abdel-Hafez and El-Maghraby[15] found that aflatoxin B_1 was produced by 7 isolates out of 146 of *A. flavus* from coffee beans in Egypt, Al-Kolaibe[3] reported that aflatoxins were produced by *A. flavus* (9 isolates out of 125 tested) collected from coffee fruits and green coffee beans from Yemen. Nakajima *et al.*[71] observed that aflatoxins B_1 produced by *A. flavus* isolated in a limited number of green coffee bean samples. Levi[72] isolated 13 aflatoxin-producer of *A. flavus* strains from green coffee beans. Ochratoxins A was produced by 8 isolates of *A. ochraceus* which were collected from coffee fruit samples from Yemen[3] and Nakajima *et al.*[71] also found 8% of *A. ochraceus* isolates from Yemeni, Tenzanian and Indonesian coffee beans samples. Abd-Alla *et al.*[23] recorded that 8% of *A. ochraceus* isolates collected from

Table 8: Mycotoxins produced by fungí isolated from coffee seeds.

	AUMC No.	Source		Toxins	Quantity U g/ 50 ml medium
		Sample No.	Locality		
Aspergillus flavus	5177	11	Khawlan, Yemen	Aflatoxin B1	450 µg
Aspergillus flavus	5178	27	Yemen	Aflatoxin B1	450 µg
Aspergillus flavus	5179	12	Harari, Ethiopia	Aflatoxin B1	450 µg
Aspergillus flavus	5180	19	Yemen	Aflatoxin B1	750 µg
Aspergillus flavus	5181	22	Yemen	Aflatoxin B1	450 µg
Aspergillus melleus	5182	2	Yemen	Ochratoxin A	600 µg
Aspergillus ochraceus	5183	11	Khawlan, Yemen	Ochratoxin B	400 µg
Aspergillus sclerotioniger	5184	1	Yemen	Ochratoxin B	400 µg
Aspergillus terreus	5185	14	Yemen	--	--
Aspergillus ustus	5186	23	Harari, Ethiopia	--	--
Aspergillus ustus	5187	27	Yemen	--	--
Aspergillus versicolor	5188	25	Harari, Ethiopia	Sterigmatocystin	280 µg
Paecilomyces variotii	5189	12	Harari, Ethiopia	--	--
Paecilomyces variotii	5190	29	Yemen	--	--
Penicillium funiculosum	5191	1	Yemen	Penicillic acid	720 µg
Penicillium funiculosum	5192	2	Yemen	Penicillic acid	240 µg
Penicillium glabrum	5193	4	Yemen	--	--
Penicillium islandicum	5194	3	Yemen	Penicillic acid	480 µg
Penicillium islandicum	5195	8	Harari, Ethiopia	--	--
Penicillium oxalicum	5196	5	Yemen	--	--
Penicillium chrysogenum	5197	21	Khawlan, Yemen	--	
Penicillium chrysogenum	5198	18	Yemen	--	--
Penicillium chrysogenum.	5199	20	Wild plants, Yemen	--	--
Trichoderma harizianum	5200	17	Yemen	Trichodermin	360 µg
Trichoderma harizianum	5201	30	Yemen	--	--

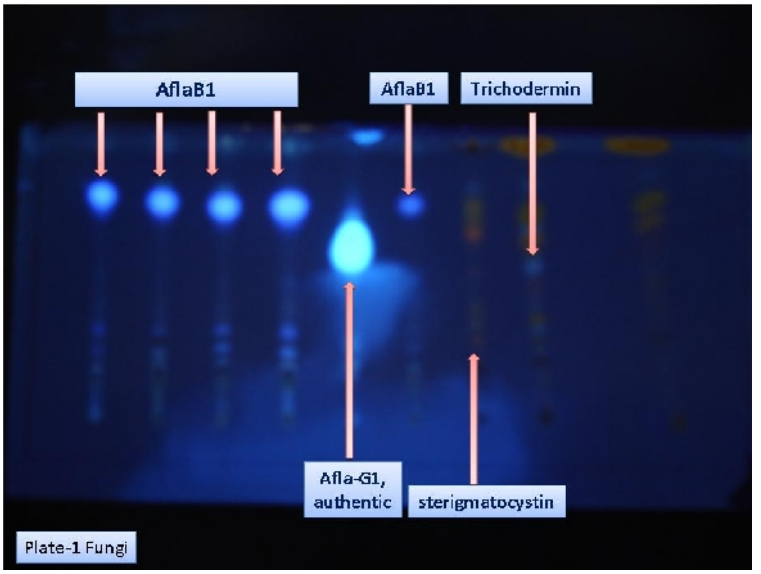

Fig. 1: Mycotoxins in plate No. 1.

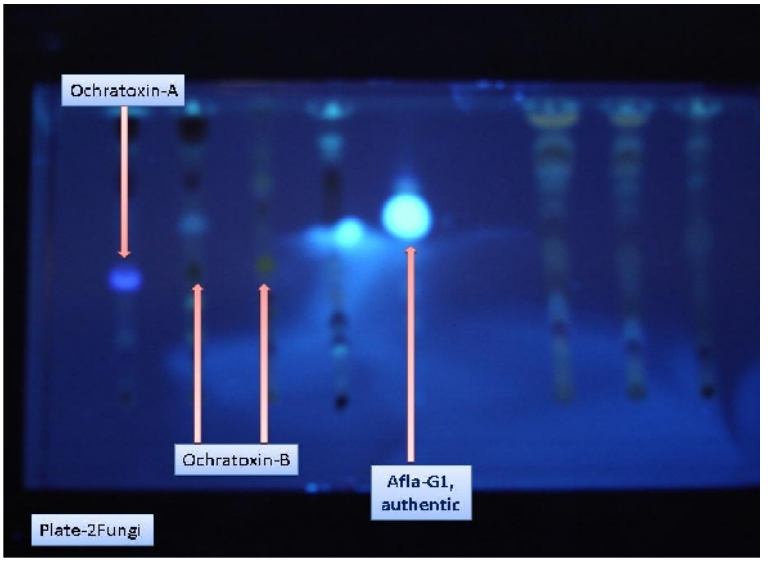

Fig. 2: Mycotoxins in plate No. 2.

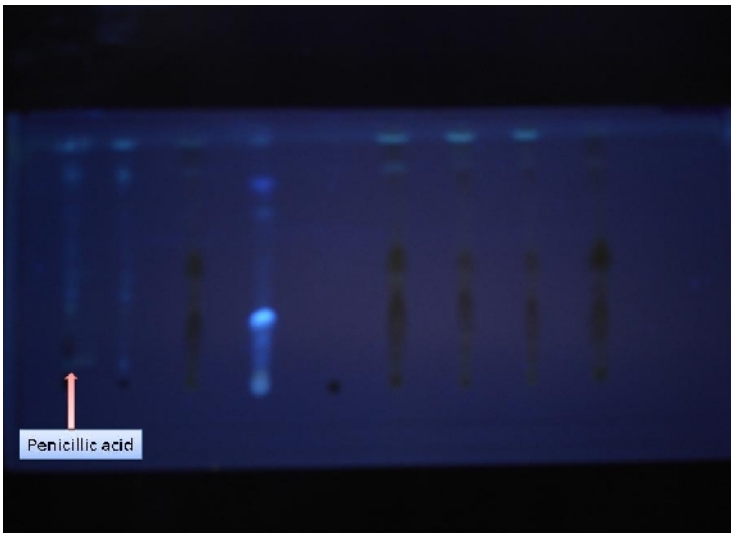

Fig. 3: Mycotoxins in plate No. 3.

green coffee beans were ochratoxins A producers. Teren *et al.*[12] found that *A. ochraceus* was responsible for ochratoxin A contamination in green coffee beans. Urbano *et al.*[74] observed that 88% of *A. ochraceus* isolates collected from Brazillian green coffee bean samples were ochratoxins A producers. Tsubouchi *et al.*[61] found that strains of *A. ochraceus* isolated from green coffee beans produced high levels of ochratoxins A. Sterigmatocystin was produced by *A. versicolor* from coffee seeds samples and this agrees with previous findings[75]. Abd-Alla *et al.*[23] observed that 6 isolates out of 51 of *A. versicolor* tested formed sterigmato-cystin from coffee beans. Also, Levi[72] recorded 9 isolates of *A. versicolor* isolated from green coffee beans were sterigmatocystin producers. Paul and Thurm[76] and Lepom and Kloss[77] found that *A. versicolor* isolated from foods could produce sterigmatocystin. Al-Kolaibe[3] reported that one isolate out of 21 tested of *A. sydowii* produced sterigmatocystin isolated from coffee bean samples.

Penicillic acid was produced by one isolate in this study (Fig. 3), while Al-Kolaibe[3] found that 2 species of *Penicillium* isolated from coffee beans did not produce mycotoxins. Indeed, great numbers of *Penicillium* spp. Have been reported to be capable to produce mycotoxins in synthetic liquid media[78-80]. Pencillic acid was recorded as antimicrobial agents and causing rat cancer[46&52]. Trichodermine was produced by *Trichoderma harizianum* in this study; Trichodermine exhibited highly cytotoxic activities[81].

Bacteriological analysis

A small survey had been conducted on coffee bean samples

Thirty-one coffee bean samples were collected from different markets at El-Riyadh, Saudi Arabia (Table 1) and analyzed Bacteriologicaly for indication of overall microbial quality by the Standard Plate Count (SPC) and *E. coli* analysis, Feacal coliform analysis and for the presence of coagulase-positive *Staphylococcus*, *Bacillus cereus* and *Salmonella* sp. Organisms. All analysis were performed on each sample. The results of bacteriological testing except for the SPC which is given in Table (9). The results indicated that the colony forming units per gram (cfu/g) unless specified and the highest count (18x104 cfu/g) was obtained from the sample No. 19 from Yemen, while the less count appeared in sample No. 18 (19x10 cfu/g), also from Yemen. *Bacillus cereus* was detected in 6 samples at levels ranging from (2x10 cfu/g) on sample No. 27 from Yemen and (6x10 cfu/g) on sample No. 11 and 24 from Harare, Ethiopia. *Feacal coliform* was detected only in one sample with the less count (4x10 cfu/g) on sample No. 7 from Yemen. *E. coli* was detected in 2 samples only (14x10 and 89x10 cfu/g), samples No. 7 and No. 23 from Yemen and Harare, Ethiopia respectively. *Staphylococcus* appeared on all samples except 2 only No. (30 and 22) from Yemen. Jan Obeta and Abriba[82] isolated *Staphylococcus epidermidis* and *S. aureus* and other bacterial species rotting of egusi. The highest count ($55x10^3$ cfu/g) was detected on sample No. 20 from Wild plants, Yemen and the lowest count (6x10 cfu/g) was detected from samples No. 18 and 29 from Yemen. Silva *et al.*[83], observed that the microbial load varied from $3x10^4$ to $2.2x10^9$ cfu/cherry, and the most common members of the genera *Aeromonas, Pseudomonas, Enterobacter* and *Serratia*. Several workers occurred on isolation of bacterial species from fruits and beans, from fermenting coffee[84], from fermentation of cacao beans[26&79] and from ripe olive[84]. All the samples used in the current study were free from *Salmonella* sp. (Table 9). Petra and Sara[85] reported that sesame seed extract and tomato also had adhesive capacities for *E. coli* kgg, *Salmonella enterica*.

PCR assay for detection of bacterial species

The amplification and detection were carried out for the tested samples, using the universal primers ITSI and ITS4[31] originated a fragment of a proximately 600 bp. This results is in accordance to the literature[86]. Analysis of the nucleotide sequences of the amplified fragments allowed the identification of the isolates at the species level (Table 2). Different-sized fragments were selected for sequencing by automated dideoxy sequencing (ABI Prism 7000 sequence detection system), using a DNA-sequence kit from Applied Biosystems. Using the DNAMAN software with the Assembly option, different fragments of each bacteria

Table 9: Bacterial isolates from 31 coffee bean samples.

Sample	TPC	TCC	FCC	Staph.	B.cereus	Salmonella
1	100x10	-	-	19x10	-	-
2	48x10	-	-	16x10	-	-
3	67x10	-	-	88x10	-	-
4	76x10	-	-	20x10	-	-
5	$68x10^3$	-	-	$17x10^3$	-	-
6	97x10	-	-	32x10	-	-
7	$60x10^3$	14x10	4x10	$14x10^3$		
8	$21x10^2$	-	-	$30x10^2$	-	-
9	60x10	-	-	11x10		
10	100x10	-	-	67x10	-	-
11	$14x10^2$	-	-	$39x10^2$	6x10	
12	$28x10^3$	-	-	$32x10^2$	3x10	
13	$43x10^2$	-	-	$25x10^2$	-	-
14	82x10	-	-	34x10	-	-
15	$12x10^3$	-	-	$43x10^2$	11x10	-
16	$53x10^3$	-	-	$49x10^3$	-	-
17	60x10	-	-	11x10	-	-
18	19x10	-	-	6x10	-	-
19	$18x10^4$	-	-	$17x10^3$	-	-
20	$11x10^3$	-	-	$55x10^3$	-	-
21	$62x10^3$	-	-	$14x10^3$		
22	$80x10^2$	-	-	-	3x10	-
23	$56x10^3$	89x10	-	$20x10^2$	-	-
24	$42x10^2$	-	-	40x10	6x10	-
25	$58x10^3$	-	-	$61x10^2$	-	-
26	65x10	-	-	14x10	-	-
27	$28x10^3$	-	-	$5x10^3$	2x10	-
28	$18x10^2$	-	-	9x10	-	-
29	$45x10^2$	-	-	6x10	-	-
30	56x10	-	-	-	-	-
31	$11x10^3$	-	-	$12x10^2$	-	-

TPC= Total Plate Count.
TCC= Total coliform count.
FCC= Faecal coliform count.
Staph= *Staphylococcus* count.
B. creus= *Bacillus cereus* count.
Salmon= *Salmonella* count.

species or gene were aligned to obtain complete sequence. Sanger *et al.*[87] recorded the fragments of ITSI-5.85-ITS2 amplification were sequenced by PCR. Primer and Probe sequences of bacteria species used in the current study were observed in Table (2). The results indicated that all samples were negative for *Yersinia enterocolitica*, *Campylobacter jeujeni*, and *Listeria monocytogenes*. While the results of *Bacillus cereus* and *Salmonella* sp. were similar to results obtained from cultural methods (Tables 1 and 9 & Figs. 4 and 5).

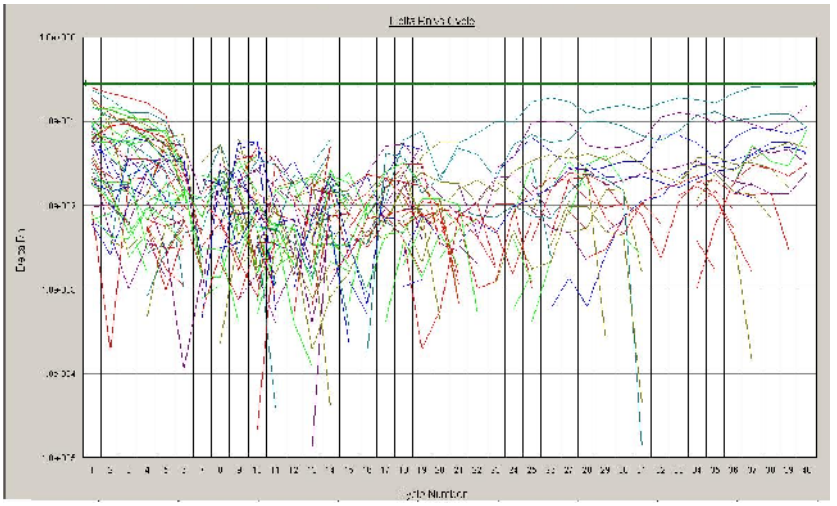

Fig. 4: PCR sequences of Data Bank.

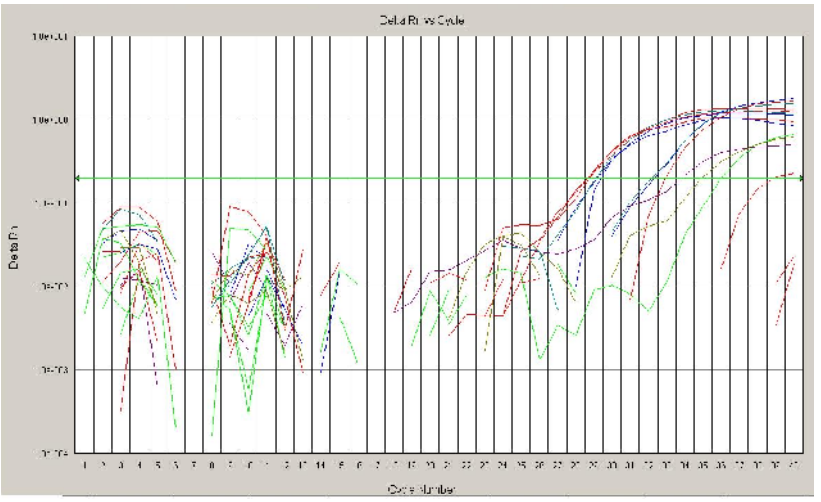

Fig. 5: Sequences of some bacterial isolates.

Conclusion

This study recorded that all coffee bean samples tested were highly contaminated by fungi and 35.5% of them were naturally contaminated by one of the two mycotoxins (aflatoxins and sterigmatocystin). Also, numerous of fungal isolates isolated from this substrate were able to produce the characteristic mycotoxins. The importance of the presence of these fungal spores is act as a source of many dangerous mycotoxins, which in small amount can be harmful to human health. Once formed, mycotoxins are stable and may remain in beans long after fungus has died. Also, coffee bean samples tested were contaminated by bacteria for example *Staphyllococcus* cotaminated all samples tested except two, but some samples were cotaminate with *Bacellus cereus*, coliform and feacal coliform. Hence, precautions must be adopted during storage to avoid their contamination by mycoflora, mycotoxins and bacteria.

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