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MYCOFLORA AND MYCOTOXINS OF CORNED BEEF

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الفلورا الفطرية وسموم الفطريات اللحم البقري المحفوظ

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أمكن في هذا البحث عزل ٦٠ نوعاً بالإضافة إلى ٥ أصناف تنتمي إلى ٢١ جنساً فطرياً وذلك من ٢٠ عينة من اللحم البقري المحفوظ (البولبيف) والتي جمعت من أماكن مختلفة بجمهورية مصر العربية . كان متوسط التعداد الكلي للفطريات يتراوح ما بين ٥٦ - ٢٣٦ ، ٤٧ - ٢٧٥ و٣٩ - ٢٠٢ مستعمرة فطرية لكل جرام واحد بولبيف على الأوساط الغذائية تشابكس اجار ، تشابكي اجار المزود بتركيز ١٥٪ كلوريد الصوديوم على التوالي . وكان الاسبرجيلس نيجر ، الاسبرجيلس فلافس والبنسيليوم كريزوجينم اكثر الأنواع الفطرية شيوعا على الأوساط الغذائية الثلاثة .

هذا وقت ثبت تلوث ١٥٪ من العينات المختبرة بسموم اوكراتوكسين (A) بتركيز يتراوح ما بين ٥٨ - ١٩٥ ميكروجرام لكل كيلوجرام بينما وجد الزيرالينون بتركيز يتراوح ما بين ٣٣٠ - ٤٢٥ ميكروجرام لكل كيلوجرام بولبيف بمستخلص عينتين فقط من كل العينات المختبرة .

وفي محاولة لانتاج سموم الافلاتوكسين معملياً على العينات الخمسة عشر الخالية من السموم الفطرية طبيعياً وجد ان جميعها قابلة للإصابة بالفطر وانتاج السم وذلك عند التحضين عند ٢٨ درجة مئوية عشر عينات فقط انتج عليها السم عند التحضين عند ١٠ درجة مئوية .

key Words : Corned beef, Mycoflora , Mycotoxins.

ABSTRACT

Sixty species and 5 varieties belonging to 21 genera of fungi were collected from 20 corned beef samples . The average total counts of fungi ranged from 56 -236, 47-275 and 39-202 colonies /g on glucose - 10% NACl - glucose - and 15% NACl - glucose - Czapek's agar media , respectively . *Aspergillus niger, A. flavus* , and *Pencillium chrysogenum* were the most common species on the three tested media . *A. fumigatus* and *A. sydowii* were common on glucose -Czapek's medium only while A. speluneus and Eurotium cheveliere were common on the media fortified by NACl . Ochratoxine A was detected in 15% of the samples at concentrations ranged between 58 and 195 mg/kg . zearalenol (330 & 425 mg/kg) was recorded in two samples only of all the tested samples . All the fifteen -mycotoxine free-corned beef samples tested proved to be susceptible to aflatoxine accumulation when inocubated at 28 C for 10 days . However, only 10 samples proved to be contaminated by aflatoxine when the infected samples refrigrated at 10 C for 30 days .

INTRODUCTION

Fungi are of ubiquitous distribution and regarded more or less a source of contamination of corned beef leading to spoilage and/or food-borne mycotoxins. Owing to the role played by fungi , whether from economic or public health point of view , advanced countries considered mould and yeast counts as a standard test for checking general sanitary conditions [1] .

The natural occurence of mycotoxines (include well known hepatotoxic, multagenic carcinogenic and teratogenic agents) in corned beef has become of inceasing interest

because of the wide spread use of corned beef in the world today . The present investigation was designed to study : a) The distribution , composition , density and occurrence of mycoflora in 20 corned beef samples . b) The natural occurence of mucotoxines in the same samples . c) The production of aflatoxins in corned beef samples which are mycotoxine-free.

MATERIALS AND METHODS

Collection of samples . Twenty samples of corned beef were collected from shops and markets at Assiut, Al-Giza and Cairo Governorates in Egypt. These samples represented the major brands of imported canned corned beef avilable for sale in Egypt . Samples were transferred to the laboratory and kept in a deep freezer (-20 C) for fungel determination and mycotoxines analysis. The samples were examined with 1- 5 days .

Determination of fungi . This was made by using dilution plate method as described by Christensen [2] . Twenty gm. of corned beef were comminuted for 1.5 min in 100 ml of .012% sterile plain agar. Further dilutions were made and one ml of an approperiate final dilution was placed in each Petri-dish. Glucose - Czapek's and 10%, 15% NACl Glucose - Czapek's agar media were used for isolation of glucophilic and xerophilic fungi , respectively . Streptomycin (20 ppm) and rose bengal (30 ppm) were used as becteriostatic agents [3] . Fifteen plates were used for each sample (5 plates for each type of media). The plates were incubated at 28°C for 7-10 days during which the developing colonies were counted, identified and the numbers were calculated per gm. corned beef in every sample.

Identification. Fungal isolates were purified by subculturig in new agar dishes and identified on the basis of their macro- and microscopic characteristics [4, 5, 6, 7, 8, 9] . Occasionally identification of fungal species was done from the culture of the origional Petri-dish .

Aflatoxin production on corned beef samples. Twenty-five g of each sample of mycotoxine - free corned beef samples were placed in sterile polythylene bag . Each bag was inoculated by 2 ml of heavy spore suspension (10^6 spores/ml) of highly aflatoxin producer *Aspergillus flavus* IMI 89717 (obtained from the International Mycological Institute , Kew, England) . The infected samples were incubated at 28°C for 10 days and referigrated at 10 C for 30 days .

Mycotoxin extraction . mycotoxins were extracted from corned beef samples according to the method described by bullerman *et al.* [10].

Clean up of the crude extracts . The dry crude extract was suspended in 50 ml chloroform and applied to silica gel column (200 mesh, MERCK) according to the method described by AOAC [11] . The column was washed with 150 ml n-hexane, followed by 50 ml diethyl ether and toxins were eluted with 150 ml of chloroform to 1 ml.

Detection of mycotoxins . Chromatographic analysis of the chloroform extracts was achieved on precoated silica gel plate type 6o F_{254} (MERCK) for the presence of aflatoxins B1,B2,G1 & G2 , citrinin , ochratoxins, patulin , sterigmatocystin, rubratoxins , T-2 toxins, diacetoxyscirpenol, zearalenon and zearalenol according to standard procedures [12 & 13] .

Quantative determination and confirmation of aflatoxins . Aflatoxin determination and confirmation were made according to the method described by Jones [14] and przybylski [15] . The sensitivity limit was about 5 µg/kg and the recovery rate about 80% .

Quantative determination and confirmation of ochratoxin A. Quantative measurement and confirmation of ochratoxin A were made according to the methods described by Borce [16] and Nesheim *et al* . [17] .

Quantitative determination and confirmation of zearalenol . zearalenol determination and confirmation were made according to the methods described by Eppley [18] and Sarudi [19] .

Source of mycotoxin standards . All of mycotoxin standards used throughout this study were purchased from Makor Chemical Ltd., Jerusalem, and kindly provided by Prof. . I . A El - Kady (Department of Botany, Faculty of science, Assiut University, Egypt) .

RESULTS AND DISCUSSION

Glucophilic fungi (recovered on glucose - Czapek's agar at 28°C) . Thirty - five species in addition to 3 species varieties belonging to 15 genera of fungi were recovered from corned beef on plates of glucose - Czapek's agar at 28 c (Table 1) . Many of these fungi had been isolated previously, but in different numbers and frequencies, from meat and meat products in Egypt [20 & 21] as well as from meat and meat products in different places of the world [22 & 23] .

The average total counts of glucophilic fungi in corned beef fluctuated from 56 - 236 colonies / g . Abdel - Rahman *et al* . [20] examined 60 samples of meat products (20 of each of minced meat, luncheon and pastirma) and recorded that the average total mould count per gm varied from 8x 103 - 100x103, 100 - 63x103 and 350 - 43x103 colonies, respectively .

Aspergillus was the most common genus in corned beef and found in all samples comprising 83. 9% of the gross total count . It was represented by 8 species and two varieties of which *A . niger, A. flavus, A. fumigatus* and *A. sydowii* were isolated in high frequency of occurrence . *A. terreus* and *A. flavus* var . columnaris were recorded with moderate frequency of occurrence . *A. terreus* and *A. flavus* var . *co-*

lumnaris were recorded with moderate frequency of occurrence while the other 3 species and one variety were isolated with less frequencies as listed in table (1) . These results are in harmony with those recorded by several researchers . Kamel *et al* . [24] found that *A. niger* was the most common species on meat followed by *A. fumigatus* and *A. flavus* . Abdel - Rahman *et al* . [20]) found that Aspergillus was the most common genus on 60 samples of meat products and identified *A. niger, A. flavus, A. fumigatus, A.sydowii* and *A.terreus* from this genus

Penicillium was the second most common genus, it occurred in 95% of the samples comprising 9 . 9% of total fungi . It was represented by 9 species of which *P . chrysogenum* was recorded in 60% of the samples . *P . oxalicum* was isolated with moderate frequency while the other species were isolated with low or rare frequencies (table 1) . Abdel - Rahman et al . [20] recorded that *penicillium* was the most common genus in 20 samples tested of each of minced meat, luncheon and pastirma . They isolated p . *chrysogenum, P. citrinum . P . verrucosum* and *P . funiculosum* from this genus . Abdel - Latif [25] identified 7 species and two varieties of penicillium in cooked meat of which *P . chrysogenum, P . citrinum* and *P . funiculosum* were the most prevalent .

Cladosporium (two species) and *Emericella* (two species and one variety) were isolated with moderate frequency of occurrence . *C . cladosporioides* was the most common species of the former and *E . nidulans* was the most common of the latter . These genera and species,were encountered, but with variable densities and frequencies, from meat products [20, 21&23] .

The remaining genera and species were less frequent and listed in Table (1) .
xerophilic fungi (recovered on 10 & 15% nacl - czapek's agar at 28 0 C) .

on 10 and 15% Nacl - Czapek's agar, 40 and 31 species and 3 and 2 varieties belonging to 14 and 7 genera were respectively collected from all samples of corned beef . The list of fungi on the two isolation media were similar except that the spectra of genera and species were narrower on 15% than on 10% Nacl - Czapek's agar . The average total counts of xerophilic fungi in corned beef ranged between 47 - 275 on 10% Nacl and 39 - 202 colonies / gm. on 15% Nacl medium .

Eurotium which was completely absent on glucose - Czapek's agar, was recovered in high frequencies of occurrence on 10 and 15% Nacl media . It was represented by 6 species and one variety of which *E . chevalieri* was isolated in high frequency on 10% Nacl and in moderte frequency on 15% Nacl media . *E . amstelodami, E . chevalieri* var . *intermedium, E . repens, E . rubum, E . pseudoglaucum* and *E . montevidense* were recorded in low or rare frequencies on the two salt media used as shown in Table (1) . The above species are well - known xerophilic; fungi [7] . Moubahser *et al.* [26] classified these species of *Eurotium* as highly xerophilic fungi, which grow on 5 - 20% or 25% Nacl .

Aspergillus (14 species and two varieties) , *Penicillium* (10 species) , *Cladosporium* (4 species), *Fennellia* (one species) and *Alternaria (A. alternata* and unidentified species) were isolated in high or moderate frequencies on the two salt media . The common species of the above genera were *A. niger, A. flavus, A. speluneus, P. chrysogenum, C. cladosporioides, C. sphearospermum, Fennellia flavipes* and *Alternaria alternata* . The remaining genera and species were less frequntly encountered (Table 1). Most of these fungi had deen isolated previously, but with different numbers and frequencies, from meat products [20, 21, 22 & 23] .

Natural occurrence of mycotoxins. Chromatographic analysis showed that 15% of the samples tested were contaminated by ochratoxin A at concentrations ranged between 58 and 195 ug/kg . When farm animals are exposed to ochratoxin A contaminated feed, a portion of the ingested toxin will be retained as residues in the tissue [27] . Thus, carry-over of ochratoxin A from contaminated seed to food of animal origin is possible [28] . Survey of meat in Denmark and Sweden has revealed that tissues from 25 - 35% of pigs suffering from nephropathy contain residues of ochratoxin A [29 & 30] . Scheuer and Leistner [31] reported the presence of ochratoxin A in 14% of kidneys and 16-19% of sausage samples examined. Levels were generally low; less than or equal to 10 ug/kg. Baumann and Zimmerli [32] reported that in about 1/3 of the analyzed meat products, ochratoxin A was detectable. The concentrations of all positive samples were below 1 mg/kg. Jelinek *et al.* [33] reported that analysis of 206 samples of meat products in Yugoslavia revealed that 12% of sausage samples were contaminated by ochratoxin A at concentrations ranged from 10 to 720 ug/kg.

Zearalenol (330 & 425 ug/kg) was detected in the extract of two samples out of all samples examined. According to the available literatures this is the first report on the natural contamination of corned beef by zearalenol. Detection of zearalenol could be attributed to either feeding of zearalenol contaminated feed or using zearalenol-contaminated ingredients in processing of corned beef.

Production of aflatoxin on corned beef. To determine the ability of *A. flavus* to produce aflatoxin under conditions of refrigeration, 15 samples (proved to be mycotoxin-free) of corned beef were inoculated with 2 ml of heavey spore suspension of *A. flavus* IMI 89717 and refrigerated at 10°C for 30 days. For comparison, the same samples were inoculated with the same toxigenic mould and incubated at 28°C for 10 days. Data obtained clearly show that all the samples tested proved to be susceptible to aflatoxin accumulation when incubated at 28°C for 10 days. Total aflatoxin concentration ranged between 55-360 ug/kg (Table, 2). Surprisingly, 10 out 15 samples proved to be con-

taminated by aflatoxins (6-12 ug/kg) when the infected samples were refrigerated at 10°C for 30 days. These results agree to some extent with that previously recorded. Ayres *et al.* [34] reported production of aflatoxins on aged hams and sauage by toxigenic strains of *A. flavus* and *A. Parasiticus* at 20° and 30°C, but at low temperatures aflatoxins were not detected. Park and Bullerman [35] reported that substantial quantites of aflatoxins were produced on summer sausage by *A. flavus* at 15°C and 25°C. Trace levels of aflatoxins (10 to 60 ppb) were detected on some samples at 5°C. Other workers have found optimal temperatures for aflatoxin production in the range of 20 to 30°C, depending on cultural conditions [36 and several others] .. Oldham *et al.* [37] concluded that refrigerating meat products prevents detectable aflatoxins production for at least 12 days even though the meat products may have been previously contaminated by *A. flavus.*

It is generally believed that *A. flavus* and *A. parasiticus* will not grow and produce aflatoxins at temperatures below

Table 1

average total counts (ATC, calculated per g fresh weight in all samples), number of cases of isolation (NCI, out of twenty samples) and occurrence remarks (OR) of fungal genera and species isolated from twenty corned beef samples on glucose-czapek's, 10% and 15% sodium chloride glucose-czapek's agar media at 28°C

Genera and species	Czapek's agar			10% NaCl Czapek's agar			15% NaCl Czapek's agar		
	ATC	NCI	OR	ATC	NCI	OR	ATC	NCI	OR
Gross total count	2338			2273		1891			
Aspergillus	1962	20	H	1351	20	H	1189	19	H
A. niger van Tiegh.	687	20	H	523	19	H	327	19	H
A. flavus Link	558	19	H	387	19	H	379	17	H
A. fumigatus Fresen.	580	19	H	158	4	L	17	2	R
A. sydowii Thom & Church	61	11	H	3	1	R	7	2	R
A. speluneus Raper & Fennell	-	-	-	112	14	H	233	10	H
A. flavus var. *columnaris.* Raper & Fennell	31	5	M	78	11	H	58	3	L
A. terreus Thom	22	7	M	13	5	M	9	3	L
A. tamarii Kita	13	3	L	6	1	R	9	1	R
A. versicolor (Vuill.) Tirab.	-	-	-	19	4	L	2	1	R
A. wentii Wehmer	-	-	-	19	4	L	9	3	L
A. alutaceus Berkeley & Curt.	2	1	R	12	3	L	5	2	R
A. janus Raper & Thom	-	-	-	2	1	R	29	3	L
A. oryzae (Ahlb.) Cohn	-	-	-	15	2	R	60	3	L
A. proliferans Smith	-	-	-	-	-	-	35	3	L
A. aureolatus Munt. Cvet. & Bata	4	2	R	-	-	-	10	1	R
A. terreus var. *aureus* Thom & Raper	2	1	R	-	-	-	-	-	-
A. fumigatus var. *ellipticus* Raper & Fennell	-	-	-	4	1	R	-	-	-
Aspergillus species	2	1	R	-	-	-	-	-	-
Penicillium	231	19	H	284	17	H	312	15	H
P. chrysogenum Thom	104	12	H	111	8	M	237	14	H
P. oxalicum Currie & Thom	32	5	M	12	2	R	-	-	-
P. citrinum Thom	24	4	L	139	9	M	62	6	M
P. brevicompactum Dierckx	21	3	L	12	2	R	6	1	R
P. janczewskii Zaleski	35	3	L	-	-	-	-	-	-
P. funiculosum Thom	3	2	R	-	-	-	-	-	-
P. corylophilum Dierckx	8	1	R	-	-	-	-	-	-
P. puberulum Bainier	2	1	R	3	1	R	-	-	-
P. purpurogenum Stoll	2	1	R	-	-	-	-	-	-
P. aurantiogriseum Dierckx	-	-	-	1	1	R	-	-	-
P. capsulatum Raper & Fennell	-	-	-	3	1	R	-	-	-
P. paxilli Bainier	-	-	-	3	1	R	-	-	-
P. jensenii Zaleski	-	-	-	-	-	-	4	1	R
P. verruculosum Peyronel	-	-	-	-	-	-	3	1	R
Eurotium	-	-	-	324	17	H	186	14	H
E. chevalieri Mangin	-	-	-	161	11	H	106	7	M
E. amstelodami Mangin	-	-	-	81	3	L	9	3	L
E. chevalieri var. intermedium (Thom & Raper) Malloch & Cain.	-	-	-	15	3	L	2	1	R
E. repens de Bary	-	-	-	38	3	L	24	1	R
E. rubrum Konig, Spiekermann & Bremer	-	-	-	2	1	R	11	3	L

Table. Cont.

Genera and species	Czapek's agar			10% NaCl Czapek's agar			15% NaCl Czapek's agar		
	ATC	NCI	OR	ATC	NCI	OR	ATC	NCI	OR
E. pseudoglaucum Blochwitz	-	-	-	27	1	R	16	1	R
E. montevidense (Talice & Mackinnon) & cain.	-	-	-	-	-	-	18	1	R
Cladosporium	39	8	M	235	16	H	179	13	H
C. cladosporioides (Fr.) de Vries	24	6	M	86	10	H	22	3	L
C. sphaerospermum Penzig	-	-	-	138	9	M	149	9	M
C. herbarum (Pers.) Link ex S.F. Gray	15	2	R	11	2	R	-	-	-
C. spongiosum Berk. & Curt.	-	-	-	-	-	-	8	1	R
Emericella	21	6	M	6	3	L	-	-	-
E. nidulans (Eidam) Vuillemin	16	4	L	6	3	L	-	-	-
E. nidulans var. *lata* (Thom & Raper) Subram.	2	1	R	-	-	-	-	-	-
E. quadrilineata (Thom & Raper) Benjamin	3	1	R	-	-	-	-	-	-
Alternaria	11	4	L	18	5	M	-	-	-
A. tenuissima (Kunze ex Pres.) Wiltshire	7	3	L	-	-	-	-	-	-
A. alternata (Fries) Keissler	4	2	R	9	2	R	-	-	-
Alternaria species	-	-	-	9	3	L	-	-	-
Fennellia flavipes Wiley & Simmons	5	3	L	18	5	M	9	2	R
Rhizopus stolonifer (Ehrenb.) Lindt	6	3	L	-	-	-	-	-	-
Scopulariopsis	17	3	L	12	4	L	14	3	L
S. brumptii Salvanet-Duval	5	1	R	8	3	L	-	-	-
S. halophilica Tubaki	-	-	-	3	1	R	14	3	L
S. candida (Gueguen) Vuill.	7	2	R	1	1	R	-	-	-
S. brevicaulis (Sacc.) Bain.	5	1	R	-	-	-	-	-	-
Syncephalastrum racemosum (Cohn) Schroeter	6	2	R	7	3	L	-	-	-
Trichoderma hamatum (Bon.) Bain.	28	2	R	-	-	-	-	-	-
Acremonium strictum W. Gams	2	1	R	-	-	-	-	-	-
Chaetomium globosum Kunze	1	1	R	-	-	-	-	-	-
Cochliobolus australiensis (Tsuda & Ueyama) Alcorn	2	1	R	-	-	-	-	-	-
Gibberella intricans Wollenweber	4	1	R	2	1	R	-	-	-
Trichothecium roseum (Pers.) Link	1	1	R	-	-	-	-	-	-
Mucor hiemalis Wehmer	-	-	-	4	1	R	-	-	-
Paecil0myces Variotii Bainier	-	-	-	3	1	R	-	-	-
Phoma glomerata (Corda) Wollenweber & Hochapfel	-	-	-	1	1	R	-	-	-
Wallemia sebi (Fr.) V. Arx	-	-	-	2	1	R	-	-	-
Nectria haematococca Berkeley & Brown	-	-	-	-	-	-	2	1	R
Mycelia sterilia (white and dark colour)	2	1	R	6	2	R	-	-	-

OR = Occurrence remarks, H = High occurrence, from 10-20 cases (out of twenty), M = Moderate occurrence, from 5-9 cases, L = Low occurrence, 3 or 4 cases, R = Rare occurrence, 1 or 2 cases

13°C [38]. However, van Walbeek *et al.* [39] reported that a strain of A. flavus produced aflatoxins at 7.5° and 10°C in 4 weeks . Also, kiermeier and Behriger [40] reported aflatoxins formation in moistened milk powder at temperatures between 1° and 5°C. Alvarez-barrea *et al* . [41] reported production of aflatoxin B1 (1.4 ug/kg) in a sample of unsmoked sausage held at high relative humidity and inoculated by *A. flavus* NRRL 6549 after 6 week at 10°C.

The data in Table (2) clearly show the effect of temperature on the ratio of aflatoxin B to G. The ratio of aflatoxin B to G was quite different at 28°C than at 10°C. Aflatoxins B1 and B2 predominated at 28°C while G1 and G2 predominated at 10°C. These results agree with those obtained by Bullerman *et al* . [42], who reported that meat infected by two toxigenic strains of *A. flavus* (NRRL 2999 and NRRL A-16100) and stored at 20°C developed high level of aflatoxins (630 µg/gm), the major portion of which (580 µg/gm = about 92%) was aflatoxin G_1, but at 30°C the mould produced equal amounts of B_1 and G_1 .

Table 2

production of aflatoxins, (ug/kg) by *A. flavus* IMI 89717 on aflatoxin-free corned beef incubated at 28oC for 10 days and 10°C for 30 days .

Sample number	28°C for 10 days				10°C for 30 days				
B1	B2	G1	G2	Total	B1	B2	G1	G2	Total
1	25	15	15	5	60	0.0	0.0	2	4	6
2	25	20	15	5	65	0.0	0.0	4	2	6
3	25	20	5	5	55	0.0	0.0	3	3	6
4	30	15	10	5	60	0.0	0.0	6	0.0	6
5	95	80	40	25	240	2	2	4	4	12
6	55	40	25	20	140	0.0	0.0	4	2	6
7	25	15	10	10	60	0.0	0.0	0.0	0.0	0
8	40	20	20	0.0	80	0.0	0.0	2	4	6
9	140	70	80	70	360	0.0	0.0	8	4	12
10	60	30	20	10	120	0.0	0.0	4	2	6
11	50	20	10	0.0	80	0.0	0.0	0.0	0.0	0
12	30	15	10	5	60	0.0	0.0	0.0	0.0	0
13	40	20	15	5	80	0.0	0.0	0.0	0.0	0
14	180	80	60	40	360	0.0	2	8	2	12
15	50	30	30	10	120	0.0	0.0	0.0	0.0	0

Although aflatoxins were found in low amounts (6-12 µg-kg) in corned beef kept at 10°C, it is not possible to determine whether the amounts of aflatoxins recovered are sufficient to cause human aflatoxicosis . van Walbeek *et al.* [43], however reported illness in a child who had consumed spaghetti containing 12.5 µg aflatoxin/kg .

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