Journal of Basic & Applied Mycology (Egypt) 5 (2014): 61-73

Mycobiota contaminating beef burger and sausage with reference to their toxins and enzymes

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Received: 6/3/2013,
Accepted: 28/12/2013

Abstract: Forty samples of beef burger and sausage (20 for each) were collected from supermarkets in Assiut Governorate during the period from July to November 2011. Samples were mycologically analyzed using dichloran Rose Bengal Chloramphenicol (DRBC) and dichloran 18% glycerol (DG18) agar media. The total number of fungal species on DRBC was higher than that on DG18 (46 versus 31 species in case of beef burger and 41 versus 33 in sausage). In the individual samples the fungal load varied from 6 - 600 colonies/g whereas the number of fungal species fluctuated between 2 and 8 species. *Aspergillus*, *Penicillium* and yeasts were the most prevalent fungi contaminating 70-100 % of the samples. *Aspergillus niger*, *A. terreus*, *A. flavus*, *Penicillium chrysogenum* and *P. citrinum* were the most common mould species on both beef burger and sausage samples. Sequencing of rRNA gene revealed the identification of 12 species belonging to 6 genera of yeasts which comprised *Candida parapsilosis*, *Galactomyces candidum*, *Pichia kudriavzevii* and *Trichosporon domesticum* as common ones. Testing the natural occurrence of mycotoxins showed that diacetoxyscirpenol and zearalenone contaminated 25 and 5 % of beef burger samples respectively whereas aflatoxin B_1 was found in only 10 % of sausage samples. Out of 24 fungal species isolated from both substrates 10 (40 %) were able to produce detectable quantities of mycotoxins. Aflatoxin B_1 was detected in the extracts of *A. flavus* cultures while aflatoxin B_1, B_2, G_1 and G_2 were produced by three isolates of *A. parasiticus*. Sterigmatocystin was formed by one isolate of *Emericella nidulans* whereas fumonisin B_1 was secreted by two isolates of *F. verticillioides*. Most fungal isolates were able to produce lipolytic and proteolytic enzymes with the most active belong to *A. parasiticus* and *F. oxysporum*, which were also toxinogenic.

Key words: beef burger, sausage, moulds, yeasts, mycotoxins, lipase, protease

Introduction

Moulds play an important role in spoilage and deterioration of meat and meat products through production of proteolytic and lipolytic enzymes. Once these enzymes have been formed before freezing, the deterioration process will not stop even such meat products are stored at freezing temperatures down to – 30 °C. Mycotoxins in meat and meat products may occur when animal feeds on contaminated feed, during slaughtering of animals, transportation and storage, contaminated equipments used for processing of meat products or due to other additives and spices (Scott and Kennedy 1973, Flannigan and Hui 1976 and Abdel-Rahman 1987). The most important mycotoxins in meat products are aflatoxins, ochratoxin A and zearalenone (Pitt 2000, CAST 2003 and Garcia and Heredia 2006).

Occurrence of fungi and mycotoxins in beef burger and sausage has become of increasing interest because of the widspread use of these types of processed meat as fast food in the world today. Owing to the role played by moulds and yeasts, whether from economic and public health point, several countries considered moulds and yeasts counts in addition to presence of mycotoxins as standard tests for checking general sanitary conditions. The present investigation was designed to study: **a)** the density and occurrence of filamentous fungi and yeasts in beef burger and sausage; **b)** the natural occurrence of mycotoxins in these foods; **c)** screening the potentiality of some isolated fungi for mycotoxin and enzyme production; **d)** testing the action of clove oil on the growth of some isolated fungi.

Materials and Methods

A- Collection of samples

A total of 40 samples of beef burger and sausage (20 for each) were randomly collected from different supermarkets in Assiut Governorate, Egypt during the period from July to November 2011. These samples represented the major brands of beef burger and sausage available for sale in Egypt. Samples were transferred to the laboratory and kept in a deep freezer (-20°C) for mycological, toxicological and enzyme analysis of samples until examined.

B- Mycological analysis

The dilution plate technique (Pitt and Hocking 2009) was employed to isolate the different fungi contaminating beef burger and

sausage samples. Two types of solid media, namely dichloran rosebengal chloramphenicol (DRBC) and dichloran 18% glycerol (DG18) were used as recommended by Samson *et al*. (2004). DRBC contained (per liter distilled water) peptone 5 g , glucose 10 g , KH_2PO_4 1 g , $MgSO_4.7H_2O$ 0.5 g, dichloran (0.2% in ethanol) 1ml, rosebengal 0.025 g, chloramphenicol 0.1 g, agar 15 g. DG18 was composed of (per liter distilled water) peptone 5 g, glucose 10 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, dichloran (0.2% in ethanol) 1 ml, glycerol 220 g, chloramphenicol, 0.1 g, agar 15 g. Ten plates were used for each sample (5 plates for each medium). The plates were incubated at 28±2°C for 7-10 days during which the developing colonies were counted, identified and their numbers were calculated per g sample.

C- Phenotypic Identification of fungal isolates

The morphological characteristics based on macro- and microscopic appearance of hyphae and spores were used for identification of filamentous fungi to species level. The following references were consulted for identification; Raper and Fennell (1965) and Pitt (1979), Moubasher (1993), Leslie and Summerell (2006), Domsch *et al*. (2007) and Pitt and Hocking (2009).

D- Genotypic Identification of yeast Isolates:

Molecular characterization of some yeast fungal species was done with the help of Solgent Company, Daejeon South Korea. Fungi were individually grown on yeast malt agar (YM) and incubated at 28° ± 2º C for 3 days. A small amount of fungal growth was scrapped and suspended in 100µl autoclaved distilled water in 2ml sterile vials and boiled at 100° C for 15 minutes. Cultures were sent to SolGent Company for rRNA gene sequencing. Fungal DNA was extracted and isolated using SolGent purification beads. Prior to sequencing, the ribosomal rRNA gene (also referred to as rDNA) was amplified using the polymerase chain reaction (PCR) technique in which two universal fungal primers ITS1 (forward) and ITS4 (reverse) were incorporated in the reaction mixture. Primers used for gene amplification have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The purified PCR products were reconfirmed (using size marker) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White *et al*., 1990). Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

E- Extraction and detection of mycotoxins

Mycotoxins were extracted from beef burger and sausage samples according to Bullerman *et al*. (1969) using chloroform. The same method was also employed for mycotoxin extraction from fungal cultures grown for 10 days on potato dextrose broth at 28°C. Thin layer chromatography was used for detection of mycotoxins (El-Kady and Moubasher, 1982) using chloroform: methanol (96:4) as a solvent system. TLC plates were visualized under short and long UV light. Mycotoxins were identified in comparison with appropriate reference standards as described by Zohri (1990).

F- Estimation of Lipolytic and proteolytic enzymes

Proteolytic activities of fungal isolates were tested using the method of Paterson and Bridge (1994) based on the hydrolysis of casein incorporated in the medium and distributed in test tubes (10 ml / each tube). Cultures were individually inoculated with the test fungus and incubated at 28° ± 2º C for 14 days. Data were recorded as the depth in mm of the clear zone resulting from casein digestion.

Lipolytic activities were similarly estimated using the medium of Ulman and Blasins (1974) in which tween 20 and $CaCl_2$ were incorporated. The depth in mm of visible precipitate (due to formation of crystals of calcium salt of oleic acid liberated by the lipase enzyme) was measured.

G- Sensitivity of fungal isolates to clove oil

The cup plate technique recommended by Kown-Chung and Bennett (1992) was employed using Czapek's yeast extract agar for growing fungal isolates. Clove oil (50µl) was pipetted in 5 mm cavities made in Petri plates inoculated with the test fungi. Cultures were incubated at 28° ± 2º C for 7 days after which the inhibition zones around cavities were measured in mm.

Results and Discussion

1. Fungi recovered from beef burger

A total of 51 species and 1 species variety belonging to 17 genera were recovered from beef burger samples. From these, 46 species and 1 variety belonging to 15 genera were recovered on Dichloran Rose Bengal Chloramphenicol agar (DRBC), and 31 species and 1 variety belonging to 10 genera were recovered on Dichloran 18% Glycerol agar (DG18) as shown in Tables (1 and 2).

a) On DRBC medium

Aspergillus, *Penicillium* and yeasts were the most common fungi being isolated from 95%, 70% and 100% of beef burger samples respectively. The counts of these fungi matched 65.28%, 7.77% and 19.81% of the gross total population of fungi respectively. *Aspergillus* was represented by 12 species and one variety from which *A. terreus* and *A. niger* were the most prevalent (65% and 50% of total samples matching 12.51% and 28.76% of total count respectively). *A. flavus*, *A. flavus* var. *columnaris*, and *A. barasiliensis* occurred in moderate incidence contaminating 35%, 45% and 35% of beef burger samples respectively. *Penicillium* was represented by 8 species of which *P. citrinum* and *P. purpurogenum* were moderately recovered (25% of samples for each). *Fusarium* was found contaminating 40% of beef burger samples accounting for 5.14 % of total fungal population. It was represented by *F. oxysporum*, *F. proliferatum*, *F. sterilihyphosum*, *F. subglutinans* and *F. verticillioides* which occurred in low incidence.

In the present study identification of yeasts was mainly based on rRNA gene sequencing using ITS1 forward and ITS4 reverse primers. As shown in Fig. 1, the phylogenic tree has seven clades covering 7 species of yeasts isolated from both beef burger and sausage in addition to closely related yeasts (often type strains) obtained from GenBank. Five other species were registered in the present investigation, which were recovered and identified in another work (Refaie 2013). Five species of *Candida* were identified, namely *C. zeylanoides*, *C. parapsilosis*, *C. intermedia*, *C. lusitaniae* (anamorph of *Clavispora lusitaniae*) and *C. catenulata*. Also, three species of *Pichia* (*P. anomala*, *P. kudriavzevii* and *P. caribaea*) were identified. The remaining species included *Debaryomyces nepalensis*, *Geotrichum candidum* (anamorph of *Galactomyces geotrichum*), *Trichosporon domesticum* and *Kluyveromyces marxianus*. From beef burger samples, five genera of yeasts were identified from which *Candida* appeared in 45% of total samples matching 16.08% of total fungal count. *Candida parapsilosis* occurred in 20% of samples matching 6.39% of total fungal population. The remaining genera and species of filamentous and yeast fungi occurred in low incidence and in low counts as shown in Table (2).

The total number of fungal colonies per sample ranged from 6 - 600 colonies/g of beef burger and the highest count was isolated from sample No. 14 (600 colonies/g). The number of fungal species per sample ranged from (2 – 11 species) with the highest being recovered from sample No. 12.

Ten fungal species were also isolated from beef burger on DRBC medium which are not shown in Table (2). These species are *A. clavatus*, *A. sydowii*, *F. proliferatum*, *F. sterilihyphosum*, *Mucor racemosus*, *P. funiculosum*, *Pochonia suclasporia* var. *catenulata*, *Trichoderma harzianum*, *Verticillium* sp. and *Candida tropicalis.*

On DG18 medium

Beef burger samples produced 10 genera and 32 species after culturing with the most common genera being *Aspergillus* and *Penicillium* (100% and 65% of beef burger samples). The count of *Aspergillus* was markedly higher than that of *Penicillium* representing 57.33% and 7.87% of the gross total population of fungi respectively (Table 2). The mycobiota isolated on DG18 were basically similar to those on DRBC. However, some fungi appeared only including *Eurotium chevalieri* and *E. amstelodami* which are well-known osmophilic species. *Pichia kudriavzevii* was only encountered on DG18. According to Ellis *et al*. (2007), this fungus is regularly associated with some forms of infant diarrhea and accasionally with systemic diseases. It has also been reported to colonize the gastro-intestinal, respiratory and urinary tracts of patients with granulocytopenia. Environmental isolations have been made from beer, milk products, skin, faeces of animals and birds.

Table 1: Total fungal counts (CFU/g) and number of fungal species isolated from beef burger and sausage samples, using DRBC and DG18 media.

Beef burger					Sausage				
	DRBC		DG18			DRBC		DG18	
Sample No.	Fungal count	No. of species	Fungal count	No. of species	Sample No.	Fungal count	No. of species	Fungal count	No. of species
1	90.4	8	79.2	8	21	44.4	9	12.6	6
2	112	7	88.8	4	22	132.6	8	43.2	6
3	232.8	5	57.6	3	23	129.6	9	64.2	4
4	52.2	10	24	3	24	99.6	4	48.6	7
5	107	5	39	2	25	93	7	36	5
6	78	7	107.2	4	26	97.8	6	58.8	6
7	84.4	7	78.4	4	27	64.8	7	18.6	4
8	115	4	54	6	28	31.8	7	20.4	5
9	117	4	72	7	29	61.2	5	42.6	3
10	24.4	6	13.6	5	30	40.2	6	25.8	6
11	89.2	8	56	7	31	282	5	259	6
12	77	11	42	5	32	290	5	266	6
13	375	8	290	6	33	81.6	5	42	5
14	600	9	310	6	34	81	9	48	7
15	265	2	125	2	35	138	9	96	5
16	23.4	7	4.8	2	36	161.4	5	102	6
17	144.6	9	50.4	6	37	31.2	8	20.4	3
18	158.4	10	153.6	9	38	21	9	29.4	6
19	89.4	6	126.6	3	39	33	7	24.6	5
20	6	2	145.8	3	40	38.4	10	12.6	6

Acremonium murorum and *Eurotium* sp. were also isolated from beef burger on DG18 medium (not shown in Table 2). The fungal species isolated during the present study were almost similar to those reported by many Egyptian investigators (Hefnawy 1980, Hegazi *et al.* 1992 and Roushdy *et al.* 1996) who found that *Aspergillus fumigatus, A. flavus, A. niger, A. terreus*, and *Cladosporium* sp., *Mucor* sp., *Candida* sp. and *Rhodotorula* sp. were the common species in beef burger samples. El-Kady and Zohri (2000) isolated 64 species belonging to 22 genera of fungi from 20 beef burger samples collected from Assiut, Giza and Cairo Governorates. The fungal population ranged from l64 to 528 colonies/g and the common fungi comprised *Aspergillus flavus, A. fumigatus A. niger, A. ochraceus A. terreus Eurotium chevalieri, Rhizopus stolonifer* and *Penicillium chrysogenum*. These findings came in close similarity with the data obtained in the present study.

2- Fungi isolated from Sausage samples

A total of 48 species and 1 species variety belonging to 14 genera were recovered from sausage samples (41 species, 1 variety and 16 genera on DRBC, and 33 species, 1 variety and 11 genera on DG18) as shown in Table (2).

a) On DRBC medium

The fungal genera and species isolated from sausage were almost similar to those obtained from beef burger (Table 2). The differences worthy to mention are: firstly, *A. flavus, A. foetidus, A. fumigatus* and *P. chrysogenum* were more frequently isolated from sausage (65 %, 30 % , 35 % and 60 %) than from beef burger (35 %, 5 %, 20% and 10% of samples respectively). Secondly, *A. flavus* var. *columnaris* occurred in lower incidence in sausage (15 %) than in beef burger samples (45%). Thirdly, some low existing fungi such as *F. verticillioides*, *Penicillium corylophilum*, *P. oxalicum*, *C. zeylanoides* and *Pichia anomala* appeared in beef burger but were completely missed in sausage samples.

The total number of fungal colonies per sample ranged from 21 - 290 colonies /g of sausage (Tables 1 and 2). The highest counts were isolated from samples No. 31 and 32 (282 and 290 colonies/g respectively).

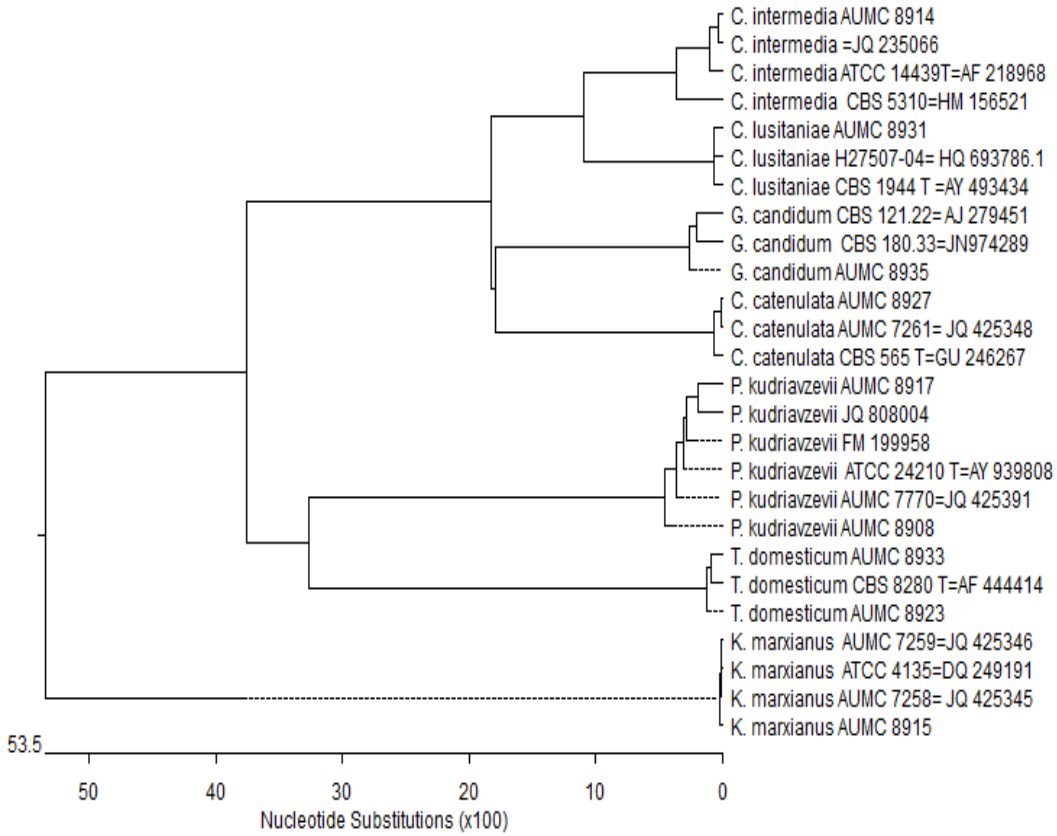

Fig 1: Phylogenetic tree for yeast species isolated from beef burger and sausage (given Assiut University Mycological Center, AUMC, Numbers), C= *Candida*, P= *Pichia*, K= *Kluyveromyces*, G= *Geotrichum* and, T= *Ttrichosporon*. The scale indicates the number of nucleotide substitutions per site.

The number of fungal species per sample ranged from 4 – 10 species with the highest number being isolated from sample No. 40 (10 species). Eight fungal species were also isolated from sausage on DRBC medium (not shown in Table 2). These species are *Emericella variecolor, Fusarium* sp., *F. solani, M. circinelloides, P. duclauxii, P. grisoefulvum, Kluyveromyces marxianus*.

b) On DG18 medium

As shown in Table (2), the majority of fungal species obtained on DG18 were of lower incidences and often in lower counts than on DRBC. Examples are *A. terreus, P. chrysogenum, P. purpurogenum, Debaryomyces nepalensis, Galactomyces geotrichum, Pichia caribaea, P. kudriavzevii* and *Trichosporon domesticum.* The total number of fungal colonies per sample ranged from 12.6 - 266 colonies/g of sausage (Table 1). Three fungal species, namely *A. tamarii, Eurotium repens,* and *P. brevicompactum* were also isolated from sausage on DG18 but not shown in Table 2.

Several investigations have been done on the mycological analysis of sausage samples both in Egypt and abroad. These studies showed variations in the fungal counts which may be due to the geographical location of producing companies, duration of storage and the hygienic status of persons dealing with these products. In mycological laboratories, the techniques used for food analysis, types of media, incubation temperature are also factors affecting the results. In Egypt**,** Lotfi *et al*. (1983) analyzed 75 sausage samples from Assiut city and found that the total mould count varied from 100 to 300 colonies per gram. Hegazy *et al*. (1992) found that the average fungal count was $9.5x10^3$ colonies/g with the dominant fungi being *A. flavus*, *A.niger, A. ochraceus* and *Cryptococcus* sp. Hassan and Ragheb (1996) reported that the mean total fungal count was 3800 colonies/g of the examined 40 samples of sausage. The incidence of species of *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Mucor*, *Rhizopus*, yeast, and *Geotrichum* was 75.0%, 32.5%, 2.5%, 5.0%, 10.0%, 2.5%, 52.5% and 15.0% respectively.

Roushdy *et al*. (1996) recorded that the mean mould count was 1.3 x 10^6/g of fresh sausage and the common species were *A. niger* and *A. flavus*, *A. ochraceus*, *P. verrucosum* and *Cladosporium* sp. El- Maraghy and Zohri (1996) isolated 73 species and four varieties belonging to 27 genera of filamentous fungi from 20 sausage samples. Their results show great similarity with the data of the present study where the average total counts of fungi fluctuated between 156 and 606 colonies/g of sample and the prevalent species were *Aspergillus niger*, *A. flavus*, *A. ochraceus*, *Penicillium chrysogenum*, *A. fumigatus* and *Gibberella fujikuroi*. Barr *et al*. (2004) analyzed 25 samples of sausage and obtained somewhat higher counts of molds ($5.87x10^3$ colonies/g) and the common genera were *Aspergillus* and *Penicillium.* Other genera such as *Alternaria*, *Cladosporium*, *Fusarium*, *Mucor*, *Rhizopus*, *Sporotricum* and *Thamnidium* were also recovered but in low percentages. El-Tabiye (2006) examined 10 samples of frozen sausage and obtained 2.9 x 10^2 CFU/g for moulds and yeasts. The isolated genera of moulds were *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, *Cladosporium* and *Mucor*. In Portugal, Matos *et al*. (2007) isolated *Penicillium, Aspergillus*, *Fusarium* and *Rhizopus* from Portuguese dry-smoked sausages.

Several investigations have been carried out on yeast contamination of sausage. Reports from UK (Dalton *et al*. 1984) revealed the isolation of 383 yeast strains from unsulphited and sulphited sausages, skinless sausages and minced beef. They found that *Debaryomyces hansenii* was the most commonly-isolated yeast followed by *Candida zeylanoides* and *Pichia membranaefaciens*. In Spain, Encinas *et al.* (2000) investigated the yeast populations on 24 lots of Spanish fermented sausages, made by four factories. They were able to identify 41 yeast strains which belong to *Debaryomyces hansenii*, *Trichosporon ovoides*, *Yarrowia lipolytica* (perfect form of *Candida lipolytica*), *C. intermedia, C. parapsilosis, C. zeylanoides* and *Citeromyces matritensis* (teleomorph of *C. globosa*).. In Egypt, Mahmoud and El-Taher (2001) studied the yeast contamination of 30 samples of sausage collected from supermarkets, shops and street vendors and the mean counts were 46 x 10^3 CFU/g for sausage samples collected from supermarkets. Samples collected from shops showed mean values of $410x10^3$ CFU/g whereas street vendor samples had average values of 4500 x 10^3 CFU/g. *Candida*, *Torulopsis*, *Rhodotorula*, *Trichosporon* and *Saccharomyces* were isolated with different perecentages. El-Tabiye (2006) obtained $1.8x10^2$ colonies/g of sample and the isolated yeasts were *Saccharomyces*, *Candida* and *Torulopsis*.

To the best of our knowledge, the following yeast species have not been previously isolated from beef burger and sausage samples in Egypt, namely *C. catenulata, C. intermedia, C. lusitaniae, C. zeylanoides, Debaryomyces hansenii, Geotricum candidum, Pichia anomala, P.caribaea, P. kudriavzevii, Ttrichosporn domesticum.*

3- Natural occurrence of mycotoxins in beef burger and sausage samples

Results in Table (3) showed the presence of diacetoxyscirpenol in low levels (less than 50 µg /kg) in five samples of beef burger. According to the available literatures, this is the first report on the natural contamination of beef burger by diacetoxyscirpenol in Egypt. Also, zearalenone occurred in a high level (more than 100 µg /kg) in only one sample of beef burger. Aflatoxin B_1 occurred in low level in two sausage samples. In this respect, Hegazy *et al*. (1992) reported that 40% of sausage samples were contaminated with aflatoxins B1 and G1 at levels of 0.75 and 0.6 mg/kg respectively. El-Maraghy and Zohri (1996) recorded that 5% and 50% of sausage samples were contaminated by zearalenone and aflatoxins B_1, B_2, G_1 & G_2, whereas 10 and 65% of beef burger samples were contaminated by zearalenone and aflatoxins at levels of 280-310 and 6-28 µg/kg respectively (El-Kady and Zohri 2000). Aflatoxin B_1 and zearalenone were encountered in 25% of tested samples of frozen meat and 12.5% of beef burger (El-Mossalami 2010). The presence of these mycotoxins in beef burger and sausage could be attributed to using meat from animals fed with mycotoxins-contaminated feed or other ingredients (e.g. spices).

Table 2: Counts (colonies/g in all samples) and frequencies of occurrence of fungal genera and species isolated from beef burger and sausage samples.

Genera and species	Beef burger						Sausage					
	DRBC			DG18			DRBC			DG18		
	TC	%TC	F&OR	TC	%TC	F&OR	TC	%TC	F&OR	TC	%TC	F&OR
Aspergillus	1855	65.16	19 H	1099.6	57.33	20 H	1301.6	66.65	20 H	866.2	66.77	20 H
A.barasiliensis Vara, Frisvad & Samson	190.6	6.71	7 M	88.2	4.59	7 M	236.4	12.11	5 M	73.2	5.64	5 M
A. candidus Link	1.2	0.04	2 L	0.4	0.02	1 L	4.8	0.25	2 L	6	0.46	1L
A. flavus Link	38.2	1.34	7 M	83.2	4.33	7 M	117.6	6.02	13 H	80.4	6.19	13 H
A. flavus var. *columnaris* Raper & Fennell	207.8	7.31	9 M	206.2	10.75	10 H	9.6	0.49	3 L	19.8	1.53	4 L
A. foetidus Thom & Raper	109.2	3.84	1 L	109.2	5.69	1 L	66	3.38	6 M	27.6	2.13	5 M
A. fumigatus Fresenius	28.4	0.99	4 L	6.6	0.34	1 L	19.2	0.98	7 M	7.2	0.56	3 L
A. niger van Tieghem	817	28.76	10 H	487	25.39	10 H	779.4	39.92	9 M	608.2	46.89	8 M
A. ochraceus Wilhelm	2.2	0.08	3 L	6.6	0.34	3 L	1.2	0.06	2 L	3	0.23	1 L
A. parasiticus Speare	74.2	2.61	2 L	16.6	0.86	2 L	11.4	0.58	2 L	13.8	1.06	2 L
A. terreus Thom	355.4	12.51	13 H	65.6	3.42	9 M	56	2.87	13 H	25.8	1.99	9 M
A. versicolor (Vuillemin) Tiraboschi	20	0.7	1 L	30	1.56	1 L	0	0	0	0.6	0.05	1 L
Cladosporium	43.2	1.52	4 L	13	0.68	2 L	2.4	0.12	2 L	4.2	0.32	3 L
C. cladosporioides (Fresenius) de Vries	41	1.44	3 L	13	0.68	2 L	1.8	0.09	1 L	1.8	0.14	1 L
C. sphaerospermum Penzig	1	0.04	1 L	0	0	0	0	0	0	2.4	0.19	2 L
Cladosporium sp.	1.2	0.04	1 L	0	0	0	0.6	0.03	1 L	0	0	0
Emericella	2.8	0.1	2 L	0	0	0	3.6	0.18	2 L	0	0	0
E. nidulans (Eidam) Vuillemin	2.8	0.1	2 L	0	0	0	0.6	0.03	1 L	0	0	0
Eurotium	0	0	0	4.2	0.22	2 L	1.2	0.06	1 L	7	0.54	2 L
Eurotium sp.	0	0	0	3.6	0.19	1 L	0	0	0	0	0	0
E. chevalieri Mangin	0	0	0	0.6	0.03	1 L	1.2	0.06	1 L	4	0.31	2 L
Fusarium	146	5.14	8 M	9.6	0.5	2 L	31.8	1.63	7 M	10.2	0.79	3 L
F. oxysporum Schlechtendal	0.4	0.01	1 L	0	0	0	13.8	0.71	4 L	4.2	0.32	2 L
F. subglutinans (Wollenweber & Reinking) Nelson *et al.*	45	1.58	4 L	9	0.47	1 L	6	0.31	1 L	0	0	0
F. verticillioides (Saccardo) Nirenberg	91.6	3.22	4 L	0.6	0.03	1 L	0	0	0	0	0	0
Penicillium	220.8	7.77	14 H	153	7.87	13 H	93.4	4.78	19 H	138.2	10.65	16 H
P. aurantiogriseum Dierckx	28.2	0.99	4 L	12.8	0.67	1 L	7.8	0.4	2 L	18.6	1.43	2 L
P. chrysogenum Thom	16	0.56	2 L	11.6	0.61	4 L	33	1.69	12 H	43	3.31	9 M

Genera and species	Beef burger						Sausage					
	DRBC			DG18			DRBC			DG18		
	TC	%TC	F&OR	TC	%TC	F&OR	TC	%TC	F&OR	TC	%TC	F&OR
P. citrinum Thom	40	1.41	5 M	101.8	5.31	4 L	26.4	1.35	7 M	48.8	3.76	7 M
P. corylophilum Dierckx	1.2	0.04	1 L	7.4	0.49	3 L	0	0	0	0	0	0
P. oxalicum Currie & Thom	4.4	0.15	2 L	6	0.31	1 L	0	0	0	0	0	0
P. pinophilum Hedgcok	1	0.04	1 L	0	0	0	1.2	0.06	1 L	0	0	0
P. purpurogenum Stoll	128.8	4.53	5 M	11.4	0.59	4 L	20.2	1.03	8 M	25.4	1.958	4 L
Rhizopus	5.2	0.18	3 L	0	0	0	3.6	0.18	3 L	1.2	0.93	1 L
R. oryzae Went & Prinsen Geerligs	3	0.11	2 L	0	0	0	0.6	0.03	1 L	0	0	0
R. stolonifer (Ehrenberg) Vuillemin	2.2	0.08	2 L	0	0	0	3	0.15	2 L	1.2	0.09	1 L
Yeasts	563.4	19.81	20 H	637.6	33.24	17 H	509	26.07	20 H	270.2	20.83	18 H
Candida	456.8	16.08	9 M	380.2	19.82	7 M	129.6	6.63	7 M	106.8	8,23	8 M
C. catenulata Diddens & Lodder	5.2	0.18	2 L	1.2	0.06	1 L	73.8	3.78	2 L	55.2	4.26	2 L
C. intermedia (Ciferri & Ashford) Langeron & Guerra	9	0.32	1 L	19	0.99	1 L	12	0.55	1 L	0.6	0.05	1 L
C. lusitaniae van Uden & do Carmo-Sousa	0	0	0	0	0	0	10.2	0.52	1 L	4.2	0.32	1 L
C. parapsilosis (Ashford) Langeron & Talice	181.6	6.39	4 M	253.8	13.23	4 L	33.6	1.72	3 L	46.8	3.6	4 L
C. zeylanoides (Castellani) Langeron & Guerra	256	9.01	2 L	106.2	5.53	1 L	0	0	0	0	0	0
Debaryomyces nepalensis Goto & Sogiyama	3.6	0.13	2 L	0	0	0	91.2	4.67	3 L	46.8	3.6	2 L
Galactomyces geotrichum (Butler & Petersen) Redhead & Malloch	5	0.18	3L	1.2	0.06	1 L	51.6	2.64	3 L	27.6	2.12	2 L
Kluyveromyces marixianus (Hansen) van der Walt	0	0	0	0	0	0	4.2	0.15	1L	0	0	0
Pichia	39.4	1.39	3 L	85.8	4.47	4 M	69	2.61	5 M	45.6	3.51	3 L
P. anomala (EC Hansen) Kurtzman	35.8	1.26	2 L	11.8	0.62	2 L	0	0	0	0	0	0
P. caribaea Phaff *et al.*	3.6	0.13	2 L	0	0	0	25.2	1.29	2 L	12	0.92	1 L
P. kudriavzevii Boiden *et al.*	0	0	0	74	3.86	2 L	43.8	2.24	4 L	33.6	2.59	2 L
Ttrichosporn domesticum Sogita *et al.*	18	0.63	2 L	6.8	0.35	1 L	22.8	1.17	3 L	3	0.23	1 L
Unidentified yeasts	42.4	1.49	4 L	49.6	2.59	3 L	140.6	7.2	6 M	40.4	3.11	4 L
Gross total count	2841.2			1918			1952.6			1297.2		
No. of genera	15			10			15+1			11		
No. of species	46+1			31+1			41+1			33+1		

TC = Total count, **%TC** = percentage total count, **F =** Frequency of occurrence out of 20 samples, **%F** = percentage Frequency, **OR** = Occurrence Remark, **H** = High Occurrence= 10-20 cases, **M** = Moderate Occurrence= 5-9 cases, **L** = Low Occurrence= 1- 4 cases, Fungi isolated from 1 or 2 samples were omitted from the table and mentioned in the text.

Table 3: Natural occurrence of mycotoxins in beef burger and sausage samples

Meat products	No. of tested Samples	No. of positive sampless	Mycotoxins	Levels*
Beef burger	20	5	Diacetoxyscirpenol	L
		1	Zearalenone	H
Sausage	20	2	Aflatoxin B1	L

***** Mycoyoxin level: H= High level (more than 100 µg /kg) and L= Low level (less than 50 µg /kg).

4- Mycotoxins produced by fungi isolated from beef burger and sausage samples

Twenty-four fungal isolates from beef burger and sausage samples belonging to *Aspergillus* (16 isolates of 4 species), *Emericella* (two isolates of *E. nidulans*) and *Fusarium* (6 isolates of two species) were tested for mycotoxin formation (Table 4). Two out of the four isolates of *A. flavus* were able to produce aflatoxin B_1. *A. flavus* AUMC 8959 produced higher levels of aflatoxin B_1 (more than 500 µg/l) than *A. flavus* AUMC 8960 (less than 100 µg/L). Both strains were isolated from beef burger (Table 2). Out of the four tested isolates of *A. parasiticus*, three showed the ability to produce aflatoxins B_1, B_2, G_1 & G_2 with moderate or low levels (Table 4). On the other hand, all tested isolates of *A. niger* and *A. ochraceus* could not produce any detectable amount of mycotoxins. These findings are in accordance with the observations of Varma and Varma (1987) who found that three out of seven *A. flavus* isolates have the ability to produce aflatoxin B_1. El-Maraghy and Zohri (1988) recorded the production of aflatoxins by two out of four tested isolates of *A. flavus* var. *columnaris*. Sanchez-Herrvas *et al.* (2008) reported that 64% of 120 tested isolates of *A. flavus* had the ability to produce aflatoxin B_1. Abdel-Kareem (2010) found that 13 out of 27 isolates of *A. flavus* had the ability to produce aflatoxin B_1 or B_1 & B_2.

In the present study, sterigmatocystin was produced by one of the two tested isolates of *Emericella nidulans*. This confirms the previous reports of Zohri and Ismail (1994) and Abdel- Kareem (2010) who extracted sterigmatocystin from *E. nidulans* recovered from meat products. Two out of the four tested isolates of *Fusarium verticillioides* was recorded as fumonisins producers (Table 4). Fumonisins are possibly carcinogenic to humans, and according to the International Agency for Research on Cancer, they are rated as class 2 B carcinogens (WHO, IARC, 1993).

5- Lipases and Proteases produced by fungal isolates from beef burger and sausage samples

The same 24 fungal isolates were tested for their ability to secrete lipases and proteases, which are very important factors for meat products spoilage. Results in Table (5) show that 23 and 21 of the 24 tested strains were able to secrete proteases and lipases respectively. It is worthmentioning that all protease-producing isolates exhibited high degree of enzyme activity, while 5, 11 and 5 of lipases-producing isolates showed high, moderate and low degrees of enzyme activity respectively. Abdel-Rahman and Saad (1989) studied the proteolytic activity of some prevalent mould species of *Penicillium*, *Mucor*, *Cladosporium* and *Aspergillus* isolated from meat and meat products and found that all species were gelatin liquefactors. *Mucor* and *Cladosporium* spp. showed the highest proteolytic activity. They concluded that the proteolytic enzymes produced by such moulds can diffuse deeply in the meat even when it is frozen. Banwart (1989) pointed out that species of *Aspergillus, Penicillium, Fusarium, Cladosporium, Geotrichum, Mucor, Alternaria* and *Rhizopus* could produce lipolytic and proteolytic enzymes.

6- Effect of Clove oil on mycelial growth of fungi

Results in Table (6) showed that clove was highly effective against 66.6% (16 out of 24) isolates and was of moderate or low activity against the remainders.

Essential oils from spices are known to possess antimicrobial activities and their efficacy as preservatives is often greater and safe than some chemical preservatives. Clove oil has shown to have strong antimycotic properties (Bullerman *et al*., 1977). El-Kady *et al*. (2000) reported that clove oil caused complete inhibition of the growth of *A. flavus* IMI 89717 and consequently aflatoxin formation when incorporated into the growth

medium or added to minced meat. Recently, Aboul-Nasr *et al.* (2014) found that clove oil completey inhibited the growth of different isolates of *C. cladosporioides* and *Stachybotrys elegans* and had a highly inhibitory action against isolates of *A. flavus*, *A. fumigatus*, *A. niger*, *Fusarium solani* and *F. oxysporum*.

Conclusion: all beef burger and sausage samples tested were contaminated with different species of moulds and yeasts. Variations in densities and kinds of these fungi are often related to the conditions of processing and storage. Most fungal isolates were able to produce lipolytic and proteolytic enzymes, which cause deterioration of these products and affect palatability and nutritional value. When mycotoxins such as aflatoxins, sterigmatocystin and fumonisins are produced, the product is considered unsafe for human consumption. Therefore care should be taken to avoid fungal contamination during processing, handling and preservation of these products.

Table 4: Mycotoxins produced by fungal isolates grown on PD at 28°C for 7 days.

Fungal isolates	Source	Fungal Biomas g/50 ml	Mycotoxins	
			Kind of mycotoxin	Levels*
Aspergillus flavus AUMC 8959	Beef burger	0.2977	Aflatoxin B_1	H
A. flavus AUMC 8960	Beef burger	0.3951	Aflatoxin B_1	L
A. flavus AUMC 8961	Sausage	0.3511	-	N
A. flavus AUMC 8962	Sausage	0.4012	-	N
A. niger AUMC 8939	Beef burger	0.4007	-	N
A. niger AUMC 8940	Beef burger	0.3375	-	N
A. niger AUMC 8941	Sausage	0.4101	-	N
A. niger AUMC 8942	Sausage	0.3416	-	N
A. parasiticus AUMC 8949	Beef burger	0.3914	Aflatoxin B_1, B_2, G_1 & G_2	M
A. parasiticus AUMC 8950	Beef burger	0.3381	Aflatoxin B_1, B_2, G_1 & G_2	M
A. parasiticus AUMC 8951	Sausage	0.2669	-	N
A. parasiticus AUMC 8952	Sausage	0.425	Aflatoxin B_1, B_2, G_1 & G_2	L
A. ochraceus AUMC 8967	Beef burger	0.3878	-	N
A. ochraceus AUMC 8972	Beef burger	0.4152	-	N
A. ochraceus AUMC 8973	Sausage	0.2504	-	N
A. ochraceus AUMC 8974	Sausage	0.3624	-	N
Emericella. nidulans AUMC 8978	Beef burger	0.596	Strigmatocystin	L
E. nidulans AUMC 8968	Sausage	0.265	-	N
Fusarium oxysporum AUMC 8981	Sausage	0.395	-	N
F. oxysporum AUMC 8984	Sausage	0.134	-	N
F. verticillioides AUMC 8986	Beef burger	0.1436	-	N
F. verticillioides 52 *	Beef burger	0.1412	Fumonisin	L
F. verticillioides AUMC 8987	Beef burger	0.365	-	N
F. verticillioides 54*	Beef burger	0.135	Fumonisin	L

Note: 52* and 54* are code numbers of fungal strains.
*Mycotoxin Levels: H = High level (equal to or more than 500 µg/l medium); M = Moderate level (less than 500 to more than/or equal to 100 µg/l medium; L = Low level (less than 100 µg/l medium) and N= Non-producer.

Table 5: Lipases and proteases produced by fungal isolates at 28°C for 14 days (depth of activity in mm) and sensitivity to clove oil (inhibition zone in mm)

Fungal isolates	Source	Lipases	Pro tease	Clove oil (50 µl/well)
Aspergillus flavus AUMC 8959	Beef burger	-	30 H	26 M
A. flavus AUMC 8960	Beef burger	2 L	27 H	45 S
A. flavus AUMC 8961	Sausage	19 M	22 H	38 S
A. flavus AUMC 8962	Sausage	14 M	21 H	36 S
A. niger AUMC 8939	Beef burger	-	35 H	3 R
A. niger AUMC 8940	Beef burger	15 M	-	34S
A. niger AUMC 8941	Sausage	11 M	27 H	33 S
A. niger AUMC 8942	Sausage	9 L	25 H	28 S
A. parasiticus AUMC 8949	Beef burger	-	31 H	34 S
A. parasiticus AUMC 8950	Beef burger	10 M	32 H	32 S
A. parasiticus AUMC 8951	Sausage	5 L	32 H	45 S
A. parasiticus AUMC 8952	Sausage	27 H	25 H	32 S
A. ochraceus AUMC 8967	Beef burger	12 M	38 H	32 S
A. ochraceus AUMC 8972	Beef burger	16 M	36 H	5 R
A. ochraceus AUMC 8973	Sausage	14 M	18 H	15 I
A. ochraceus AUMC 8974	Sausage	20 H	39 H	5 R
Emericella nidulans AUMC 8978	Beef burger	20 H	30 H	25 I
E. nidulans AUMC 8968	Sausage	20 M	35 H	38 S
Fusarium oxysporum AUMC 8981	Sausage	15 M	30 H	53 S
F. oxysporum AUMC 8984	Sausage	25 H	45 H	12R
F. verticillioides AUMC 8986	Beef burger	18 M	31 H	43 S
F. verticillioides 52*	Beef burger	7 L	40 H	45 S
F. verticillioides AUMC 8987	Beef burger	9 L	39 H	32 S
F. verticillioides 54*	Beef burger	14 M	36 H	26 I

Notes: 52* and 54* are code numbers of fungal strains.
Degree of activity: H= High, M= Moderate 10-19 mm, and L=Low ≤ 10 mm.
Sensitivity to clove oil: S= Sensitive (inhibition zone ≥ 30 mm), I= Intermediate (zone 15-29 mm), R= Resistant (zone less than 15 mm)

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