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Mycological evaluation of some ready to eat meat products with special reference to molecular characterization

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Abstract

Sixty samples of random meat products luncheon, basterma and hawawshi (20 of each) were collected from different localities in Kalyobia governorate, Egypt. The collected samples were subjected to mycological examination, detection of aflatoxin B_1 residues as well as the ability of isolated A.flavus for production of aflatoxin B_1 and identification of toxigenic strains by PCR. The obtained results revealed that the examined luncheon samples had the highest mould count with a mean value $4.7x10^2 \pm 2.1 x10^2$ followed by basterma samples $3.4x10^2 \pm 8.6x10$ and Hawawshi samples had lower mould count $7.9 x10 \pm 5.2 x10$. Six mould genera could be detected and identified. The identified mould genera were belonging to genus Aspergillus, Penicillium, Cladosporium, Mucor, Eupenicillium and Talaromyces. The highest incidence of isolate among Aspergillus spp. was A. niger followed by A.flavus and A.parasiticus. The average concentration of aflatoxin B_1 (µg/kg) in luncheon, basterma and hawawshi were 1.4± 0.63, 0.8 ±0.47 and 0.7± 0.36, respectively. Toxigenic A.flavus were subjected to PCR identification. Three A.flavus toxigenic isolates were examined by polymerase chain reaction (PCR) with using specific primer (PEPO1 &PEPO2). PCR products of A.flavus strains were positive on agarose gel electrophoresis of PCR amplification products showing 200 bp. The public health importance of the isolated moulds and aflatoxins and the recommended points were discussed

Keywords: meat products- mycotoxins- PCR- toxigenic strains

Introduction

Ready to eat (RTE) meat products constitutes a major share of the processed meat products. Ready-to-eat (RTE) products, such as luncheon, basterma and hawawshi are prepared to be eaten without the need for further cooking and therefore often consumed without additional cooking steps. Post-process handling is a cause of recontamination of RTE meat products especially with food pathogen. Consumers may choose to cook them for a better taste or appearance **(Ray, 1996)**.Contamination of meat products with different mould species considers a real hazard as it affecting the quality of these meat products by increasing the opportunity for its spoilage and deterioration. The most important aspect about mould spoilage of food is, however, the formation of mycotoxins. The most dangerous type of mycotoxins are aflatoxins. Aflatoxins are the main toxic secondary metabolites of some Aspergillus spp. such as A. flavus, A. parasiticus and the rare A. nomius **(Alcaide-Molina et al., 2009)**. Ochratoxin A (OTA) is a toxin naturally produced by several species of Aspergillus and Penicillium **(Aish et al., 2004)**.The aim of this study is to determine the mycological quality of some ready to eat meat products in Kalyobia supermarkets. This mycological study determines the total mould counts; isolation and identification of recovered mould species and determination of aflatoxins B_1 residues in the examined meat products as well as ability of isolated A.flavus for production of aflatoxins B_1 and identification of toxigenic strains by PCR.

Material and Methods

Collection of samples

A total of 60 random samples of basterma, luncheon and hawawshi (20 of each) was collected from different localities in Kalyobia governorate. The collected samples were kept in sterile polyethylene bags and preserved in an ice box then transferred to the laboratory under complete aseptic condition without undue delay to be examined mycologically.

Fungal counting, isolation and identification. Total fungal count was carried out according to the techniques recommended by **ISO (217-1-2:2008)**.Mould were isolated and identified according to macro and microscopic characteristics as described by **Pitt and Hocking (2009).Detection of aflatoxin B_1 residues in meat products.**The aflatoxin B_1 residues were estimated according to technique recommended by **Stubblefield et al. (1982)** and **AOAC (2000).**

Detection of aflatoxins B_1 from isolated A. flavus. The ability of isolated Aspergillus flavus strains for production of aflatoxin B_1 were

estimated according to technique recommended by **(Davis et al., 1966).**

Identification of toxigenic strains using PCR.PCR amplification were done according were carried out to technique recommended by **(Gallo et al. 2012).**The DNA was extracted with (QIAamp DNA Mini Kit, Qiagen company, Germany) using the method as described by manufacture manual as following: Genomic DNA of the strains was obtained using the genomic DNA Extraction Kit (QIAamp DNA mini kit; Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration was determined spectrophotometrically at 260/230 nm. Primer sequence were PEPO1 5′ (CGACGTCTACAAGCCTTCTGGAAA, 3′) PEPO2 5′ (CAGCAGACCGTCATTGTTCTTGTC, 3′). The PCR reaction was performed in an Gradient thermal cycler (1000 S Thermal cycler Bio-RAD USA).The reaction mixture (total volume of 50 µl) was 25 µl Dream green PCR Mix (DreamTaq Green PCR Master Mix (2X) Fermentas Company, cat., No.K1080, USA.), 5 µl target DNA, 2 µl of each primers (containing 10 p mole/ µl) and the mixture was completed by sterile distal water to 50 µl **(Logotheti et al., 2009).**

PCR amplification conditions for A. flavus were: 5 min initial step followed by 38 cycles at 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1.5 min and a final extension step at 72 °C for 5 min. Amplification products were electrophoresed in agarose gels (3% w/v) (Agarose, Sigma, USA) was used for running of DNA. Stained with ethidium bromide Using GeneRuler 100 bp DNA Ladder: Fermentas Company, Cat. No.SM0243, US

Statistical Analysis: The data were statistically treated by way ANOVA using SPSS program for windows (Version 16) (SPSS Inc. Chicago, IL and USA) and Duncan's post hoc test with $P < 0.05$ considered to be statistically significant.

Results And Discussion

According to the results illustrated in figure (1) the incidence of fungi in the examined meat product samples were 14 (70%),13(65%) and 6 (30%) for luncheon , basterma and hawawshi, respectively. The results obtained for luncheon and basterma and hawawshi were similar to that recorded by many investigators, **Brr et al. (2004), Hussein (2008), Abd-Allah and Ismail (2012), Ismail et al. (2013)** and **Morshdy et al. (2015).** Higher values were recorded by **Mousa et al. (2014)** and **Abu Zaid (2015)** .Meanwhile lower counts were obtained by **Hafez (2003)** and **Saleh and Salah El-Dien (2006).** In recent decades, the question of mould toxicity has attracted attention, especially in the fields of agriculture and food industry. Microscopic filamentous fungi often contaminate vegetal and animal products, becoming a source of diseases in man and slaughter animals **(Mižáková, et al., 2002).** The reason for an increasing interest is the ability of moulds to produce secondary metabolites – mycotoxins – that have unfavourable effects, such as carcinogenesis, mutagenicity, and high thermostability. The environment in the manufacturing rooms, stores, refrigerators and shops are very suitable for the development of moulds inside the products, but more frequently on the surface of various sorts of meat and meat products **(Jesenská, 1987).**

Table (1) revealed the total fungal count of examined meat product samples. In luncheon ranged from 2x10 to $4.2x10^3$ with a mean value of $4.7x10^2 \pm 2.1x10^2$. Basterma ranged from 1x10 to $1.2x10^3$ with mean value of $3.4\ x10^2 \pm 8.6x10$. Hawawshi ranged from1x10 to $1x10^3$ with mean value of $7.9x10 \pm 5.2x10$.From obtained results, the presence of fungal contamination may be attributed to the use of contaminated spices (untreated food additives) which usually carry mould spores used in manufacture of luncheon, basterma and hawawshi. Also, it was found that the heat treatment used in luncheon and hawawshi during processing affect the fungal spore, resulting in decrease the mould contamination in these products .While the low contamination level of mould obtained in basterma samples may be referees to the low water activity (w_a) in this product and also presence of garlic which act as antifungal **(Morshdy et al., 2015).**The difference associated with the examined sample of meat products were highly significant ($P<0.001$) as a result of average total mould counts as shown in **table (2).** As seen in table (3) various moulds were detected in luncheon, basterma as well as in hawawshi examined samples. The most frequently isolated genera were Aspergillus spp., Mucor spp. and Penicillium spp.Ten species of moulds have been isolated from various meat products. The incidence of identified mould isolated from examined luncheon, basterma and hawawshi were (20, 15and 5 %) for Aspergillus flavus, (25, 40 and 15 %) for Aspergillus niger, (0, 5 and 0%) for Aspergillus parasciteus, (15, 5 and 5 %) for

Penicillium decumens, (0, 15 and 0 %), for Penicillium citrinum, (5, 0 and 10%) for Penicillium corylophilum, (0, 5 and 0 %) for Cladosporium spp.,(10,0 and 0%),for Eupenicillium spp.,(15,15 and 25%) for Mucor spp., (0,0 and 10 %) for Talaromyces spp. The results of mould identification agreed with those obtained by **Ouf et al. (2010), Ismail et al. (2013), Abu Zaid (2015)** and **Morshdy et al. (2015).**On the other hand, the conditions of the environment in the manufacturing rooms, stores, refrigerators and shops are very suitable for the development of moulds inside the products, but more frequently on the surface of various types of meat and meat products. Also moulds can play an important role in the spoilage of food due to production proteolytic and lipolytic enzymes, some moulds can also produce mycotoxins that can be harmful to humans.The results presented in **table (4)** showed that Aflatoxins B_1 was detected in examined luncheon, basterma and hawawshi samples with mean values of 1.4 ± 0.63, 0.8±0.47 and 0.7± 0.36 ppb, respectively. Moreover, the incidence of aflatoxin B_1 in the examined samples luncheon, basterma and hawoshi were (30%), (15%) and (15%), respectively. Nearly similar results obtained by **El-Tabiy (2006), El-Diasty and Wahba (2008), El-mossalami (2010), Ismail et al. (2013)** and **Morshdy et al. (2015).** While higher detectable levels of aflatoxins residues were reported by **Hegazi et al. (1992), Hassan and Ragheb (1996), Ismail and Zaky (1999)** and **Shaltout et al. (2014).** These different mean values of aflatoxins residues may be related to the residues of aflatoxin present in raw minced, additives used in the processing, level of additives contamination with aflatoxin. At the same time, the mean values of detected aflatoxins in the examined samples were lower than the maximum permissible limit recommended by WHO "15 ppb" **(Jelinek et al., 1989)** and FAD limit "20 ppb" **FAO (2004).**Moreover, the results recorded in **table (5)** showed that the isolated A. flavus from meat products having the ability to produce aflatoxin B_1 with a percentage of 38.5%. The average amount which could be detected was 300 ± 63.2 µg/L media. These findings are supported by the results obtained by many investigators, for instance **El-Diasty and Wahba, (2008), Pitt and Hoching (2009)** and **Ezekiel et al., (2014).**Three A.flavus toxigenic isolates were examined by molecular methods polymerase chain reaction (PCR) with using specific primer (PEPO1 &PEPO2). PCR products of A.flavus strains were positive on agarose gel electrophoresis of PCR amplification products showing 200 bp **(Figure 2).**

Conclusions

Mould was in the meat products established momentarily and showed a high diversity of filamentous fungi mainly belonging to the genera Aspergillus, Penicillium, Cladosporium, Eupenicillium and Talaromyces. The most of the mould genera which considered as a major cause in the spoilage of meat products, leading to great economic losses and constitute a public health hazards by production of wide variety of mycotoxins. However the level of aflatoxin B_1 residues detected in the examined samples were lower than the permissible limit recommended by WHO and FAD. In general, the microbiological quality of meat products, are dependent on the quality of the raw materials, other materials used or added during processing operations, efficacy of cooking process and whole of the sanitary measures adopted.

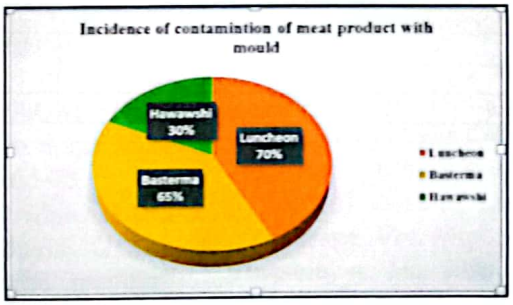

Figure (1): Incidence of contaminated meat product with mould

Table (1): Average of total mould counts (TMC/g) of meat products (N= 60).

Examined Samples	Total mould count (cfu /g)			Accepted samples	
	Min.	Max.	Mean ± SE	N0.	%
Luncheon	2x10	$4.2x10^3$	$4.7x10^2 \pm 2.1x10^2$	6	30
Basterma	1x10	$1.2x10^3$	$3.4x10^2 = 8.6x10$	10	50
Hawawshi	1x10	$1x10^3$	7.9x10± 5.2x10	13	65

Table (2): Analysis of variance (ANOVA) of total mould count in examined meat product samples (n=20).

Source of variance	Sum of square (S.S)	D.F.	M.S	F. value
Between treatment	829098.6	2	414549.3	
Residual	21699827	57	380698.7	*7.3
Total	22528925.6	59		

S.S=Sum of squares; D.F=Degree of freedom; M.S=Mean squares;*=highly significant differences betwee treatments (P<0.001)

Table (3): Prevalence of mould species isolated from examined meat products (N= 20).

Mould genera	Luncheon		Basterma		hawawshi	
	No.	%	No.	%	No	%
Aspergillus spp.						
A. flavus	4	20	3	15	1	5
A.niger	5	25	8	40	3	15
A. parasciticus	0	0	1	5	0	0
Penicillium spp						
P.decumens	3	15	1	5	1	5
P.citrinum	0	0	3	15	0	0
P.corylophilum	1	5	0	0	2	10
Cladosporium spp.	0	0	1	5	0	0
Eupenicillium spp	2	10	0	0	0	0
Mucor spp.	3	15	3	15	5	25
Talaromyces spp.	0	0	0	0	2	10

% calculated in relation to number of samples.

Table (4): Incidence and detectable level of aflatoxin B_1 residues (ppb) in examined samples (N=20)

Meat products	No. of +ve samples	%	Min.	Max.	Mean ± SE	**Accepted samples
Luncheon	6	30	2.3	10.5	1.4± 0.63	20
Basterma	3	15	3	8	0.8±0.47	20
Hawoshi	3	15	1	6	0.7± 0.36	20

S.E* = standard error of mean,**Permissible limit according to WHO "15 ppb" (Jelinek et al., 1989) and FAD limit "20 ppb" FAO (2004).

Table (5): Number of A. flavus isolates positive for toxin production (ug / L).

Strains	No. of isolated strains			Min.	Max.	Mean ± SE
	No.	+ve	%			
A.flavus	8	5	38.5	200	500	300± 63.2

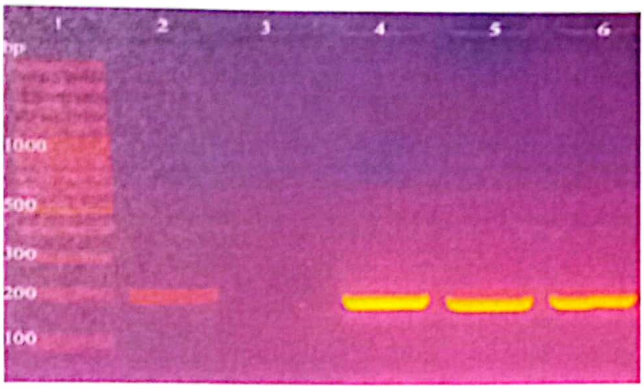

Figure (2): Single PCR performed with genomic DNA; Lane 1: 100 bp DNA ladder; Lane 2: control Positive; Lane 3: control Negative and Lane 4-6: A. flavus

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الملخص العربي

التقييم الفطري لبعض منتجات اللحوم الجاهزة للأكل مع إشارة خاصة إلى التوصيف الجزيئي

*فهيم عزيزالدين شلتوت و**رمضان مصطفي سالم **ايمان محمود الدياسطي
و*** فاطمة عبد الله حامد دياب
*قسم مراقبة الاغذية - كلية الطب البيطري - جامعة بنها
** قسم الفطريات بمعهد بحوث صحة الحيوان بالدقي
***المدينة الجامعية بجامعة بنها

لقد هدفت هذه الدراسة الى تقييم الجودة الفطرية لمنتجات اللحوم الجاهزة للاكل المتداولة في الاسواق ، مصر. حيث تم جمع ستين عينة من منتجات اللحوم بواقع 20 عينة كل من اللانشون والبسطرمة والحواوشي من مناطق مختلفة في محافظة القليوبية، مصر. أخضعت العينات التي تم جمعها للفحص للتلوث بالفطريات وكذلك للكشف عن بقايا الأفلاتوكسين ب ، في منتجات اللحوم وكذلك قدرة عترات الاسبرجليس فلافس المعزولة على إنتاج الافلاتوكسين ب١ . كان متوسط العد الكلى للفطريات بالنسبة لعينات اللانشون والبسطرمة والحواوشي٤,٧ × ١٠ ٢ ± ٢,١ × ١٠ ٢ ، ٣,٤ ×١٠ ٢ ± ٦,٨ ×١٠ ، ٧,٩ × ١٠ ± ٥,٢ × ١٠ مستعمرة/ جرام على الترتيب . تم عزل وتصنيف ستة أنواع من الفطريات. الأنواع التى تم عزلها من الفطريات اشتملت على أجناس الأسبرجيلس ، البنسيليوم ، الكلادسبوريوم، الميوكور، ايوبنسيليوم و التالروميسيس. حيث اظهرت النتائج ان الاسبرجليس نيجر كان الاعلي نسبة يلية الاسبرجليس فلافس ثم الاسبرجليس بارازتيكس. كما اظهرت النتائج ان متوسط تركيز الافلاتوكسين ب ١ في كل من اللانشون والبسطرمة والحواوشي ١,٤ ± ٠,٦٣ ، ٠,٨ ± ٠,٤٧ و ٠,٧ ± ٠,٣٦ (ميكروجرام / كجم). كما اظهرت النتائج ان متوسط نسبة الافلاتوكسين النتائج من العترات الاسبرجليس فلافس المعزولة كان ٣٠٠ ±٦٣,٢ (ميكروجرام / لتر).تم التعرف على بعض معزولات الاسبرجليس فلافس باستخدام تفاعل البلمرة المتسلسل. وتمت مناقشة الأهمية الصحية للفطريات والسموم الفطرية وعلاقتها بالصحة العامة .