PREVALENCE OF FUNGI IN LOCALLY PRODUCED CHEESE AND MOLECULAR CHARACTERIZATION OF ISOLATED TOXIGENIC MOLDS

ELbagory, A. M.[1]; Amal, M. Eid [2]; Hammad, A. M. [1]; Salwa, A. Dawood [1]
[1]*Department of Food Hygiene & Control, Fac. of Vet. Med., Sadat City University.*
[2]*Animal Health Research Institute. Tanta branch, Egypt*

A B S T R A C T

One hundred and forty samples of locally produced Tallaga, Kareish, Processed and Ras cheese (35 each) collected from dairy shops and supermarkets for mycological studies. Our results revealed that, the moulds and yeasts could be detected in all examined samples of Tallaga, Kareish, processed and Ras cheese with mean count values of $8.3x10^2 \pm 0.2x10^2$ $6.9x10 \pm 0.4x10$,$5.3x10^3 \pm 2.3x10^2$ and $4.1x10^3 \pm 1.6x10^2$ for moulds and $0.5x10^2 \pm 1.1x10$,$1.4x10^2 \pm 0.5x10^2$,$6.5x10^4 0 \pm .4x10^4$ and $5.4x10^3 1 \pm .1x10^3$ for yeasts respectively. Various types of molds and yeasts were isolated at varying percentages from all examined samples. The isolated moulds were species of genera Asprigallus, Penicillium, Cladosporium, Mucor. and Rhizopus, while yeast genera were species of genera Candida and Rhodotorula. When the obtained results compared with the Egyptian and international standards, the examined Kareish cheese samples were found to be of lower quality than the other types of cheeses examined, although all types of the present study need to be improved microbiologically. In this study, rapid assessment of five isolates of *A. flavus* was accomplished using a primer pair for the Aflatoxin regulatory gene *afl.R1* in polymerase chain reaction (PCR) and only 2 isolates obtained from processed and Ras cheese (one each of) were positive the presence of the target gene.

Key words: Tallaga cheese, Kareish cheese, processed cheese, Ras cheese, Molds

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1. INTRODUCTION

In addition to milk and its products are being nutritious food for human, they provide a favorable environment for the growth of various microorganisms. Yeasts and moulds can grow in milk and its products particulary at suitable conditions of temperature and moisture (Barrois et al., 1997). Contamination of these products may occur from the raw material or during manufacturing, storage and distribution (Kure et al., 2004). Such microorganisms influence the biochemical characters and flavor of such products as well as their appearance rendering them commercially undesirable and often resulting in decreasing the grading grading of the dairy product (Demarigny et al., 1997 and Muir & Banks, 2000). Some moulds can also adversely affect human and animal health as they can produce mycotoxins which are fungal secondary metabolites formed by consecutive series of enzyme- catalyzed reactions from a few biochemically simple intermediates of primary metabolism, these mycotoxins can enter the human and animal food chain by direct or indirect contamination (Bohra & Purohit , 2003). Some moulds are related to a range of pathologies ranged from gastroenteritis to cancer, as these mycotoxins are highly toxic, mutogenic, teratogenic and carcinogenic substances (Adams & Moss, 2000; Li et al., 2000 and Hussein & Brasel, 2001).

Aflatoxin - producing fungi belong to several Aspergillus species including A.

flavus and A. parasiticus ; the major species of concern for aflatoxin contamination , and other species like A. nomius, A. pseudotamarii, A. bombycis, A. ochraceoroseus (Cary et al. , 2005 and Frisvad et al., 2005). A. flavus is the main producer of Aflatoxins that produce aflatoxin B1, the most known potent liver carcinogen and Aflatoxin B2, (Pitt & Hocking, 1999).

Worldwide, Aflatoxins are the most important Mycotoxins in foodstuffs and they can produce acute and chronic toxicity in animals and humans. In addition, the high carcinogenicity produced by these Mycotoxins in animals justifies every effort to monitor and reduce it in foods (Pitt & Hocking, 1999). Reports on the occurrence of yeasts in cheeses were related to the early part of this century, but it is still not widely appreciated that yeasts can be an important component of many, if not all, cheese varieties (Ferreira & Viljoen, 2003). They can either cause spoilage or produce desirable biochemical changes in cheese. Spoilage of cheeses by yeasts appears as visible growth of yeast colonies on the surface of cheese, as unpleasant smell or taste, changes in color and texture and/or deformation of the packets containing the cheese (Effat, 2000). Their occurrence may be attributed to the yeast's ability to grow at low temperatures, the assimilation of organic acids like succinic, lactic and citric acid, their proteolytic and impolitic activities, resistance against high salt concentration, low α_w and the relatively resistance to cleaning compounds and sanitizers (Martin et al.,2007). Furthermore, yeasts have the ability to tolerate low pH and low water activity values (Ferreira &Viljoen, 2003).

The level of fungal contamination as well as the identification of the main species is important, since they could give an indication of the food quality as well as of the potential due the presence of mycotoxins (Suanthie et al., 2009). The development of a rapid, sensitive method for detection and differentiation of potential aflatoxigenic species in foods is needed to estimate any associated potential health risk (Valasek & Repa, 2005). Conventional methods for detection and identification of fungi in foods rely on microscopic or culture techniques, which are time consuming and laborious. Information derived from these test would allow informed decisions about storage life of the product and the need for specific Mycotoxin analysis. In this direction, DNA-based detection methods such as PCR appear more sensitive and specific.The Aflatoxins biosynthesis pathway involves approximately 25 genes clustered in a 70 kb DNA region (Yu et al., 2004). A. flavus, A. parasiticus, and other Aspergillus Sect. Flavi species share nearly identical sequences and conserved gene order in the cluster. In recent years, PCR detection of Aflatoxin biosynthetic gene presence or expression has been used as diagnostic tool for aflatoxigenic fungi in selected food commodities (Geisen, 2007). Sequence variability and deletions in various genes/regions of the aflatoxin biosynthetic cluster have also been used to determine the polyphyletic assemblage of A. flavus group / species (Chang et al., 2005 and Chang et al., 2006).

Therefore, the objective of this study was to monitor the distribution of fungi in different types of cheese commonly consumed in Egypt and identification of isolated fungal strains. Isolated Aspergillus Sect. Flavi was subjected to molecular identification to establish the presence in their genome of the characterized Aflatoxin biosynthetic gene in relation to Aflatoxin production.

2. MATERIAL AND METHODS

2.1. Collection of samples.

A total of one hundred and forty samples ; 35 each of soft cheese (Tallaga and Kareish), Processed cheese and Ras cheese were collected from dairy shops and supermarkets, all samples was transported in a sterile air tight jars to the laboratory

with a minimum of delay to be prepared and subjected to mycological examination examined .

2.2. Preparation of cheese samples

Ten grams from each sample were homogenized with 90 ml sterile 0.2 % sodium citrate solution in a stomacher bag (Lab-blender 400, Seward, UAC House Friars Road, London SE19UG. Model No. 6021). One ml from the original sample homogenate was added to a test tube containing 9 ml 0.1% sterile peptone water to provide a dilution of 10^2. Similarly a ten tenfold serial dilution were prepared (*APHA, 2003*)

2.3. Enumeration, Isolation and Identification of fungi:

The yeast and mould counts of examined samples were determined according to the technique recommended by A.P.H.A (1992). Isolated molds were identified according to Collins and Lyne, (1984), while isolated yeast were identified according to Kriger Van Rij (1984) and by using rapid miniaturized system API 20 C AUX (bioMérieux, France) according to Kurtzman et al., (2003). Isolated A.flavus strains were subjected to molecular detection of their ability to produce Aflatoxins using PCR technique.

2.3.1. Extraction of fungal DNA

DNA was extracted from 0.5 g (wet weight) fungal mycelia / spores. The mycelium/ spores were transferred to a mortar, froze in liquid nitrogen and were ground well. Steps of extraction had been completed using Spin Column DNeasy Plant Mini Kit Qiagen cat. No. 6910

2.3.2. PCR Assay

PCR primers were purchased from Chromogen Company, South Korea. PCR Master Mix: DreamTaq Green PCR Master Mix (2X) Fermentas Company, cat., No.K1080, USA) was used in this work from Fermentas company.

2.3.3. Polymerase Chain Reaction

The polymerase chain reaction was used to amplify the Aflatoxin regulatory gene fragments of aflatoxigenic fungal genomic DNA. The sequence of the forward and reverse primers *aflR1* of the Aflatoxin regulatory gene was (5¯ AACCGCATCCACAATCTCAT 3¯) and (5¯AGTGCAGTTCGCTCAGAACA 3¯), The primers that cover the region from 540 to 1338 of Aflatoxin regulatory gene with product size of 798 base pairs (bp) have been patented according to Farber et al., (1997).

The polymerase chain reaction was performed in a Gradient Thermal cycler (1000 S Thermal cycler Bio-RAD USA) was 25 µl; each reaction mixture was heated to 95ºC for 10min. A total of 30 PCR cycles, each cycle at 30 sec at 94ºC for denaturation, 45 sec at 55ºC for annealing, 1.15 min at 72ºC for extension and a 10min final extension at 72ºC. The PCR products were analyzed by electrophoresis on a 1.5% agarose (Agarose, Sigma, USA) in (1x) TBE buffer, stained in 1µ /gel ethidium bromide. Using GeneRuler 100bp DNA Ladder: Fermentas Company, Cat.No.SM0243, US. The gel visualized by trans-illuminator and photographed by digital camera

2.4.Statistical analysis:

It was conducted using the mean, standard deviation by SPSS V.16

3. RESULTS

Table (1) showed contaminated samples from Tallaga, Kareish, processed and Ras cheeses with moulds at percentages of 94.3, 100, 77.1 and 82.9 % respectively with mean values of $57.6x10^2 \pm 23.1x10^2$, $20.1x10^3 \pm 3.6x10^3$, $16.2x10^2 \pm 5x10^2$ and $84.9x10^2 \pm 68.5x10^2$ respectively.

Table (1): Statistical analytical results of molds count for examined cheese samples (Total mold count / g). N=35

Types of cheese samples	Positive samples		Cfu /g		
	No.	%	Min	Max	Mean ± S.E
Tallaga	33	94.3	10 x 10	$65x10^3$	$57.6x10^2 \pm 23.1x10^2$
Kareish	35	100	30 x 10	$80x10^3$	$20.1x10^3 \pm 3.6x10^3$
Processed	27	77.1	10x10	$10x10^3$	$16.2x10^2 \pm 5x10^2$
Ras	29	82.9	10x10	$20x10^4$	$84.9x10^2 \pm 68.5x10^2$

Table (2): Statistical analytical results of total yeast count/ g for examined cheese samples (N = 35)

Type of cheese samples	Positive samples		cfu/g		
	No.	%	Min	Max	Mean ± S.E
Tallaga	27	77.1	10 x 10	$23x10^3$	$54.6x10^2 \pm 10.8x10^2$
Kareish	35	100	10×10^2	$15x10^4$	$45.2x10^3 \pm 7.8x10^3$
Processed	12	34.3	10x10	$45x10^2$	$13.6x10^2 \pm 4.2x10^2$
Ras	27	77.1	10x10	$67x10^3$	$10x10^3 \pm 3.4x10^3$

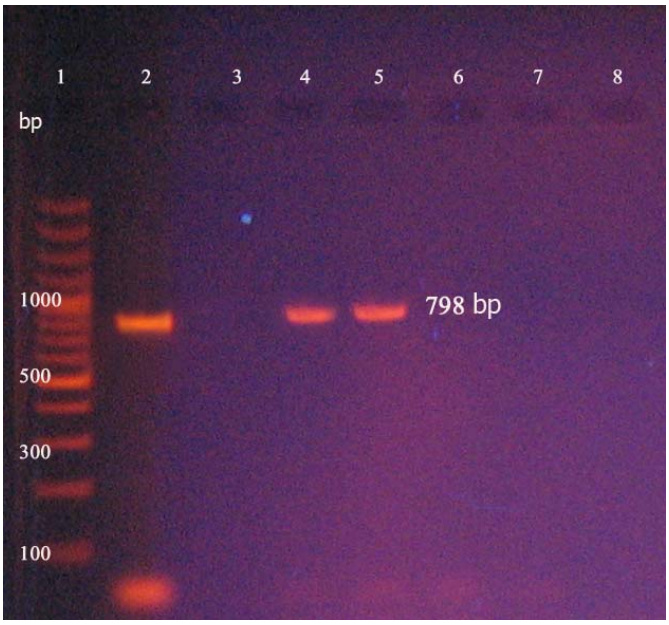
Table (3): Incidence of fungal isolates in the examined cheese samples. (N =35)

Genera & species	Tallaga		Kareish		Processed		Ras	
	NO	%	NO	%	NO	%	NO	%
Mold species								
Aspergillus species								
A. flavus	1	2.8	0	0	2	5.7	2	5.7
A. niger	0	0	0	0	0	0	3	8.5
A. fumigatus	9	25.7	10	28.5	5	14.2	14	40
A. terreus	1	2.8	1	2.8	1	2.8	3	8.5
Penicillium spp.	6	17.4	12	34.2	8	22.8	6	17.4
Fonsecaea spp.	5	14.2	7	20	6	17.4	8	22.8
Nigrospora spp.	4	11.4	0	0	0	0	4	11.4
Paecilomyces spp.	0	0	0	0	1	2.8	0	0
Scopulariopsis spp.	1	2.8	0	0	1	2.8	0	0
Mucor spp.	0	0	0	0	1	2.8	0	0
Rhizopus spp.	1	2.8	0	0	0	0	0	0
Tricoderma spp.	1	2.8	0	0	0	0	0	0
Yeast species								
Candida albicans	8	22.8	14	40	12	34.2	8	22.8
Rhodotorula spp.	1	2.8	5	14.2	1	2.8	1	2.8
Candida famata	3	8.5	5	14.2	2	5.7	1	2.8

Table (4): Quality of examined cheese samples according to *EOSQC (2005)*

Examined cheese samples	Standards according to *EOSQC (2005)*							
	Moulds ≤ 10 cfu/g				Yeast ≤ 400 cfu/g			
	Acceptable		Unacceptable		Acceptable		Unacceptable	
	No./35	%	No./35	%	No./35	%	No./35	%
Tallaga	2	5.7	33	94.3	11	31.4	24	68.6
Kareish	0	0	35	100	0	0	35	100
Processed	8	22.8	27	77.2	28	80	7	20
Rass	6	17.1	29	82.9	18	51.4	17	48.6

Figure (1): PCR-amplified products of Aflatoxin gene. Lane 1: 100bp DNA ladder. Lane 2: Control Positive. Lane3: Control Negative. Lane 4-5: positive sample. Lane 6-8: Negative sample



4. DISCUSSION

Results reported in table (1) reveals that, the examined cheeses samples of Tallaga, Kareish, processed and Ras were contaminated with moulds at percentages of 94.3, 100, 77.1 and 82.9 % respectively with mean values of $57.6x10^2 \pm 23.1x10^2$, $20.1x10^3 \pm 3.6x10^3$, $16.2x10^2 \pm 5x10^2$ and $84.9x10^2 \pm 68.5x10^2$ respectively. For Tallaga cheese nearly similar results were reported by Aman, (1990), ELsayed et al., (2011) and Hathout et al., (2013). Meanwhile higher mould counts were observed in Tallaga cheese samples examined by Ghazal (2001); Abd - ELshaheed (2004); Salih et al., (2012); and Hegazy and Mahgoup (2013). Concerning Kareish cheese samples, similar results were observed by Kaldes, (1997) and EL-Diasty & Salem (2007). Higher findings were reported by EL-Ghaish (2004). While lower findings were observed by Sayed (2011); Hosny, et al., (2011); Metwalli (2011) and Aly et al., (2012). Regarding the results of processed cheese lower incidence were observed by Palmas et al., (1999) and Hussein et al., (2011). While relatively,

higher counts were recorded by Nour-ELDiam and ELZubeir (2006) and EL-Shibiny el al., (2013). The obtained results of Ras cheese results proved to be similar to what have been reported by Sadek , et al. (2009) and EL-Leboudy et al. (2014) for the examined 3 month aged Ras cheese samples ,while a higher results was seen in the examined 6 month aged samples, Lower incidence and count were recorded by Torkar & Teger (2006). The results recorded in Table (2) pointed out that the examined samples of Tallaga, Kareish , processed and Ras cheese were contaminated with yeast at percentage of 77.1, 100, 34.4 and 77.1 % respectively with meanccount values of $54.6x\ 10^2 \pm 10.8x10^2$, $45.2x10^3 \pm 7.8x\ 10^3$, $13.6x10^2 \pm 4.2x10^2$ and $10x\ 10^3 \pm 3.4x10^3$ respectively.

Nearly Similar incidence of yeast in Tallaga cheese samples was observed by ELsayed et al., (2011), while nearly similar counts were reported by Hathout et al.,(2013), meanwhile higher results observed by Kandeel,(1993); Abd-ELshaheed (2004);Hegazy & Mahgoup (2013))and Salih et al., (2012). Regarding the results of Kareish cheese samples, comparatively similar results were obtained by EL-Diasty & Salem (2007). While higher counts were observed by EL-Shafei et al., (2008). Meanwhile lower incidence and counts were observed by ELsayed et al., (2011); Aly et al., (2012) and Hakim et al., (2013). In processed cheese, relatively lower incidence was observed by Palmas et al., (1999) and lower counts were observed by Hussein et al., (2011) and EL-Shibiny et al., (2013), however a higher result was obtained by NourELDiam and EL-Zubeir (2006). The results of Ras cheese lower incidence and counts were recorded by Torkar & Teger (2006) and higher one was observed by Sadek et al., (2009). Yeasts and moulds counts in cheese are used as an index of the proper sanitation quality. Defects in this un-ripened soft cheese such as rancidity, softness and color defects arise mainly from contamination by yeast and mould. Moreover, some species constitutes a public health due to production of mycotoxins (Rippon, 1982). The main defects caused by yeasts are fruity, bitter or yeasty off flavors, gas production, discoloration changes and texture. In fact, continued lactose fermentation could be lead to increased acidity, gassiness and fruity flavors, while continued hydrolysis of protein and fat could contribute to bitter and rancid flavors as well as a softening of product texture. (Soloiman et al., 2011). Inspection the results in Table (3) showed that in Tallaga cheese samples, species of Asperigillus as A.flavus, A. fumigatus, and A. terreus were isolated in percentages of 2.8, 25.7 and 2.8 % respectively. While Penicillium Spp was present in percentage of 17.4%. Fonsecaea Spp, Nigrospora Spp, Scopulariopsis Spp and Rhizopus Spp were detected in 14.2, 11.4, 2.4 and 2.8%, respectively. Candida albicans, Candida famata and Rhodotorula spp. were found in percentage of 22.8, 8.5 and 2.8 %. Yeasts and moulds are widely distributed as environmental contaminants of air, water, soil and dust, so the presence of moulds and yeasts in milk products may be attributed to poor sanitary practices during manufacturing, packing and distribution or the use of bad quality raw ingredients (Ray, 1996). In samples of Kareish cheese, A. fumigatus, and A. terreus, were present in percentages of 28.5 and 2.8 % respectively, similar results were observed by EL-Diasty & Salem (2007). Penicilluim Spp, Fonsecaea Spp were present in percentage of 34.2, 20 %. Candida albicans, Candida famata and Rhodotorula Spp. were present in percentage of 22.8, 14.2 and 2.8%, respectively. Nearly similar results were obtained by EL-Shafei et al., (2008). In examined processed cheese samples A. flavus, A.fumigatus, A. terreus were present in percentages of 5.7, 14.2 and 2.8 % respectively. While Penicillium, Fonsecaea, Paecilomyces, Scopulariopsis, and Mucor species were found in percentage of 22.8, 17.8, 2.8, 2.8 and 2.8 % respectively. Higher incidence of toxigenic Spp. of genus

Aspergillus was observed by Mor & Singh (2000). While Candida albicans, Candida famata and Rhodotorula Spp. were found in 34.2, 5.7and 2.8 %. In samples of Ras cheese, Asperigallus spp. were represented as A. flavus, A.niger, A. fumigatus, and A. terreus in percentage of 5.7, 40, 8.5 and 8.5% respectively. Meanwhile, Pencillum, Fonsecaea, Nigrospora species were present in percentage of 17.4, 22.8 and 11.4%, respectively. Candida albicans, Candida famata and Rhodtorula spp. were present in the percentage of 22.8, 2.8 and 2.8%. Nearly similar results were obtained by Salwa, (1999) and Corbo et al., (2001). The major sources of Rass cheese molds ,yeast were found in air, equipment and the plastic films of packaging, where air is considered as the major source of cheese contamination so high quality air with low number of contaminants in production room especially the wrapping room is important in order to reduce mould contamination (Kure et al. 2004).

As shown in Table (3) Asperigallus, penicillium, and candida species were the most common genera recovered from the four types of examined cheese samples. Similar results were recorded by Sampayo et al., (1995), Elprince & Ismail (1998) ; El-Sherif (2000) and Montagna et al., (2004). Growth of Penicillium, Cladosporium, Asperigillus and Mucor species may responsible for bitterness and rancidity of cheese. Penicillium species may lead to softness the surface of cheese Minervini et al., (2001). Some species of Asperigallus, Cladosporium, Penicillium and Fusarium were responsible for kerato-conjunctivitis in man while Asperigellus niger causes otomycosis and allergic condition, some species of penicillium have been associated with pulmonary infections, urinary tract infections and yellow rice disease and may lead to death in man Nilesen et al., (1998). According to the aforementioned results in Table (4), 94.3, 100, 77.2 and 82.9 % of the examined Tallaga, Kareish, processed and Ras cheese samples, respectively had molds above the allowed limit (unacceptable) and 68.6, 100, 20 and 48.6 % had yeasts counts above the allowed limit (unacceptable) compared with EOSQC (2005). Certain food borne yeasts & molds may be hazardous because of their ability to elect allergic reactions (Mislivec et al., 1992). Moreover, discoloration and off flavor are common defects caused by growth of fungi on cheese as well as mycotoxins production which have been associated with several cases of mycotoxicosis (Neal et al., 1998). Presence of anaerobes is indicative of careless methods of production, as an index of fecal or soil contamination and there was definite correlation between the hygienic conditions of production and the content of anaerobes in such product. It is worth to mention that, the probability of food borne illness. Several genotypic techniques have been developed in the last decades reported that genes involved in the Aflatoxin bio synthetic pathway may form the basis for an accurate, sensitive, and specific detection system, using PCR, for aflatoxigenic strains in grains and foods (Shapira et al., 1996). In this study, using primer designed to Aflatoxin regulatory pathway gene, aflR, made the presence of aflatoxigenic fungi was easily detecting in compared to conventional plating techniques. five morphologically identified A. flavus was the only species detected in connection with aflatoxin production, one from Tallaga cheese samples and 2 from both processed and Ras samples. Only two isolates were positive to this gene and gave two bands at 798 bp. Studies carried out by Hashim, et al., (2013) on PCR amplification of aflR gene in different food samples using the same primer pair did not show any false priming results due to the presence of food components or any other contamination. Such technique is able to screen many, suspected samples in a time, resource saving manner in fine and expensive products of foods with highest possible accuracy.

Therefore, the information derived from these tests would allow informed decisions about storage life of cheese and

the prevention strategies eventually needed. To improve the safety of this product, efforts to raise awareness of the importance of hygiene barriers and raw milk quality as well as improved process control can be suggested, we can recommend that the receiving of raw milk should be carefully monitored and only obtained from suppliers apply good manufacturing practices. Also strict hygienic measures of cleaning and sanitization of all food contact surfaces and hygienic training of plant workers should be applied to avoid contamination. Water supply must be clean and comply with the standard requirements, prevention of environmental contamination, good cleaning and sanitizing of food processing is essential to produce safe and high quality cheese. Added Rennet should be carefully monitored and only obtained from suppliers apply good manufacturing practices. Good conditions of air, surfaces and packaging materials through which mould spores can enter the factory and storage room environment. All these schedules are key issues in controlling mould contamination on cheese and other processed dairy products.

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كلية معتمدة 2013

مدي تواجد الفطريات والخمائر فى الجبن المنتج محليا والتوصيف الجزيئي للفطريات السامة المعزولة

عبد الرحمن محمود الباجوري[1]، امل مصطفي عيد[2]؛ احمد مصطفى حماد[1]، سلوي داوود[1]
[1] قسم الرقابة الصحية على الأغذية-كليه الطب البيطري-جامعه مدينة السادات
[2] معهد بحوث صحة الحيوان – طنطا

الملخص العربي

تم تجميع مائه واربعين عينة من مختلف أنواع الجبن، الجبن الثلاجة، القريش، المطبوخ والجبن الرومي (35 من كل منها) جمعت من محلات الالبان والسوبرماركت لدراسة المحتوي الفطري فيها. وأظهرت النتائج تواجد الفطريات والخمائر في العينات المختبرة بالمتوسطات الحسابية الأتية: $10^2\times57,6 \pm 10^2\times23,1$ ، $10^3\times20,1 \pm 10^3\times3,6$ ، $10^2\times16,2 \pm 10^2\times5$ و $10^2\times48,9 \pm 10^2\times68,5$ للفطريات علي التوالي و للخمائر كانت المتوسطات الحسابيه كالاتي: $10^2\times54,6 \pm 10^2\times10,8$ ، $10^3\times45,2 \pm 10^3\times7,8$ ، $10^2\times13,6 \pm 10^2\times4,2$ و $10\times10^3 \pm 10^3\times3,4$ علي التوالي. كما تم عزل الفطريات والخمائر الأتية من العينات قيد الدراسة بنسب مختلفة *Asprigallus spp, Penicillium spp, Cladosporium spp, Mucor spp. Rhizopus spp. Candida spp , Rhodotorula spp.* . كما هدفت الدراسة الي الكشف عن قابليه عترات *Asprigallus flavus* الخمس المعزولة لإنتاج الافلاتوكسين B1 باستخدام زوج من البوادي متخصصه لتحديد الجين المنظم لانتاج الافلاتوكسين aflR في تفاعل البلمره المتسلسل باستخدام الدنا المستخلص من المعزولات الخمسه للاسبيرجلس فليفس. هذا وقد وجدت عينتان ايجابيتان لوجود الجين (واحده في كل من عينات الجبن المطبوخ والجبن الرومي).

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