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OCCURRENCE OF AFLATOXIN-PRODUCING MOULDS IN CHEESE

(With 4 tables)

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تواجد الفطريات المفرزة لسم الأفلاتوكسين فى الجبن

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تنتشر الفطريات فى أماكن وأوساط كثيرة مما يسهل من تلويثها لكافة أنواع الغذاء وبشكل خاص الحبوب الزراعية وأيضا بعض الأغذية الحيوانية كاللبن وبعض منتجات الألبان والتى يأتى فى مقدمتها الجبن بأنواعه المختلفة. لهذا فقد أجريت هذه الدراسة على ٤ أنواع مختلفة من الجبن بواقع ٣٠ عينة من كل نوع لمعرفة مدى إحتوائها على الفطريات. وقد أثبتت الدراسة أن كلا من الجبن الرومى والجبن القريش أكثر تعرضا للتلوث بالفطريات المختلفة من نظيريهما الجبن الدمياطى والجبن المطبوخ. وأوضحت نتائج العزل والتصنيف أن فطرا الأسبرجللس والبنيسيليوم هما الأكثر شيوعا بين مختلف الملوثات الفطرية فى سائر أنواع الجبن الأربعة. كما بينت دراسة إمكانية إفراز السموم الفطرية أن ١٥من جملة ال٢٤ عترة من فطر الأسبرجللس فلافس و٨ من جملة ال١٥ عترة من فطر الأسبرجللس باراسيتيكس التى تم عزلها إيجابية عند إستخدام طريقة الفلورسنس. فى حين أنه باستخدام التحليل الكروماتوجرافى رقيق الطبقة وجد أن العترات الإيجابية من كلا الفطرين كانت ١١ و٦ فقط على الترتيب، غير أن جميعها مما أظهر إيجابية لطريقة الفلورسنس. لذلك فقد أوصينا بإمكانية إستخدام طريقة الفلورسنس كطريقة سهلة ورخيصة وبشكل خاص عند إستبعاد كل ما لا يمكنه إفراز السموم من فطر الأسبرجللس.

SUMMARY

A total of 120 cheese samples of 4 different types (30 samples each) collected randomly from different localities in Ismaillia Governorate were mycologically investigated. The average mould counts were $3.9x10^3$, $5.1x10^3$, $7.2x10^4$ and $2.3x10^2$ cfu/g as estimated from the

examined samples of Kareish, Damietta, Ras and processed cheese, respectively. Species belonging to the genera *Aspergillus* and *Penicillium* comprised the majority of mould isolates from all the 4 types of cheeses. The mycotoxigenicity of the isolated 24 strains of *A. flavus* and 15 strains of *A. parasiticus* was examined by a fluorescence technique (UV), and by thin-layer chromatography (TLC). By fluorescence technique, 15 and 8 strains of *A. flavus* and *A. parasiticus*, respectively, exhibited illumination under UV light, while only 11 and 6 isolates, respectively, gave positive results on using TLC; all of which from the illuminating group.

Key words: Moulds - Cheese - Aflatoxins

INTRODUCTION

Fungi being ubiquitous in nature can gain access to cheese at any time during manufacturing and storage. Hundreds of such food-associated mould strains proved to be able to produce toxic metabolites (mycotoxins).

Since 1961, the time at which Sargeant et al. reported what they had termed "turkey X disease" resulting from a toxic metabolite of *Aspergillus flavus*, many authors had contributed to the study of mycotoxins. Rossel and Pritchard (1991) defined mycotoxins as secondary metabolites produced by filamentous fungi and having harmful biological effects in animal and, in some cases, man. Problems with mycotoxins are great in less developed countries where storage facilities for grains and other types of foods are inadequate and where food shortage dictate the consumption of mould-contaminated food. Contamination of animal feeds by mycotoxins may result in carryover of the mycotoxins into edible animal products. Of most concern is the occurrence of aflatoxin M_1, a metabolite of aflatoxin B_1, in milk and other dairy products, including cheese (Egmond, 1983).

Concerning the public health significance and the pathogenesis of mycotoxins, it is well known that in many cases potential problems involve the possibility of cancer or delayed organ damage due to repeated ingestion of subacute levels. Aflatoxins produced by members of the *Aspergillus flavus* group have been associated with hepatitis, cirrhosis, Reye's syndrome, udorn encephalopathy and hepatoma in man. Also, in addition to long term illness, acute symptoms can result from foods

contaminated with such metabolites (Varman and Evan, 1991). Protection of the consumers from foods contaminated with such hazardous substances forced many health authorities allover the world in making strict regulations pertaining to mycotoxins in human foods, as well as animal feedstuffs (Rossel and Pritchard, 1991).

Indeed, because fungal growth is a prerequisite for mycotoxin production, this study is conducted to explore to what extent cheeses manufactured in Egypt are exposed to contamination by fungi, as well as studying the ability of some isolates in production of aflatoxins.

MATERIAL and METHODS

Sampling

A total of 120 cheese samples included Kareish cheese, Damietta cheese, Ras cheese and processed cheese (30 samples each) were collected randomly from different localities in Ismaillia Governorate under aseptic conditions. Each sample was delivered to the laboratory in a sterile wide mouthed glass stoppered sampling bottle kept at 4°C until being examined.

Determination of mould counts

The method recommended by A.P.H.A. (1992) was used. 11 grams from each cheese sample were aseptically mixed with 99 ml of sterile 2% sodium citrate solution using sterile electric stab mixer to form the dilution 1/10. The subsequent serial dilutions were carried out therein. One ml from each of the desired dilutions was transferred aseptically into duplicated sterile 15 cm diameter peteri dishes to which ~20 ml from Malt Extract Agar were mixed uniformly. Mould colonies were counted within 3-7 days of incubation at 20°C and the mould counts were calculated thereafter and expressed as colony forming units (cfu)/ g cheese.

Isolation and identification of moulds

Representative pure colonies from each morphologically identical type of mould were picked up and inoculated onto Malt Extract Agar slopes and stored at 5°C before being macroscopically and microscopically identified according to the taxonomic methods recommended by Raper and Fennell (1965&1977), Booth (1971), Samson (1979), Ramirez (1982) and Klich and Pitt (1988).

Detection of mycotoxigenicity

The mycotoxigenic activity exhibited by the isolated *A. flavus* and *A. parasitcus* strains was detected using two different methods.

A. Fluorescence of the inoculated agar medium under ultraviolet (UV) light (Hara et al, 1974)

All of the isolated *A. flavus* and *A. parasitcus* strains were inoculated at the center of solidified Czapek's solution agar in glass Petri dishes and incubated at 28°C in the dark. Plates were examined under UV illumination from the seventh through the tenth day of incubation for the presence or absence of blue fluorescence in the agar surrounding the colonies.

B. Thin-layer chromatography (TLC) according to A.O.A.C. (1970)

The isolated strains of *A. flavus* and *A. parasitcus* were inoculated into 50 ml of Yeast Extract Solution (YES) containing 20% sucrose and incubated at 25°C for 7-10 days. The aqueous phase was extracted by blending for 5 min. with 25 ml chloroform; the mixture was centrifuged, and the chloroform layer was decanted and retained. Chloroform exraction of the aqueous layer was repeated. The two chloroform fractions were combined, filtered, and concentrated to dryness using rotatory vacuum evaporator. Residues were taken up in 5 ml of chloroform. The concentrated extracts were spotted onto activated thin-layer chromatography (TLC) plates coated with 0.25 mm thickness silica gel. The concentration of the various spots was determined visually in comparison with aflatoxin standards.

RESULTS

The results are described in Tables 1, 2, 3 & 4.

DISCUSSION

It is clear from the results given in table 1 that both Kariesh and Ras cheeses were considerably massively contaminated with mould in relation to the other types; Damietta and processed cheeses. 24 out of the 30 samples constituting 80% of the examined samples of Ras cheese contained fungal growth with an average of 7.2×10^4 cfu/g. Damietta

cheese appeared to be the least contaminated one as only 13 samples (43.3%) were contaminated. Despite processed cheese had given the lowest average count (2.3×10^2 cfu/g). More or less similar findings were demonstrated by Abdel-Sater et al. (1995) particularly those concerning processed cheese and Arevalo et al. (1996). However, higher values of mould counts were assessed by Ibrahim (1987). Also, a considerable higher values of mould contamination were reported by El-Shinay and Ragheb (1995) upon examining processed cheese. Nevertheless, lower mould counts were recorded by Arizcun et al. (1996). Such variation in the obtained results by different investigators could be attributed to the hygienic conditions and type of milk used (heat-treated or not), differences in manufacturing practices, handling the raw materials and the final product and the effectiveness of hygienic measures applied at both production and storage levels.

Table 2 describes the incidence of various types of mould genera and species isolated from the examined samples of the 4 different types of cheeses. It is obvious from the tabulated data that number of cheese samples contained aspergilli and penicillia distinctly exceeds those having other types of mould genera. Also, on analyzing the data, one can conclude that both *A. flavus* and *A. parasiticus* dominated all isolates belonging to the genus *Aspergillus* followed by *A. ochraceous* and *A. niger*. This could be attributed to the fact that *A. flavus* and *A. parasiticus* are common contaminants in foods originating from the soil. Moreover, they are adapted to warmer environment prevalent in subtropical regions (Dorner et al., 1989). The higher incidence of *A. flavus* at the expense of *A. parasiticus* may be due to the common occurrence of *A. flavus* spores in air in relation to *A. parasiticus*. The later associated more frequently with soil (Diener and Davis, 1965). On the other hand, isolates belonging to the genus *penicillium* were dominated by *P. verrucosum*, while *P. rubrum* came as the least frequent one. These findings settled what has been reported formerly by El-Bassiony et al. (1980), Aman (1987), Ibrahim (1987), Hassanin (1993), Abdel-Sater et al. (1995), Abouzeid et al. (1996) and Arizcun et al. (1996). However, Bullerman (1980), El-Shinay and Ragheb (1995) and Arevalo et al. (1996) reported somewhat varied incidences of mould genera and species upon the examining cheeses.

Findings presented in table 3 show the results obtained upon testing the mycotoxigenicity exhibited by the 24 isolates of *A. flavus* and 15 isolates of *A. parasiticus* using the fluorescence of the agar medium

under UV light and TLC method. Although it is not obviously stated in the table, yet all the 11 isolates of *A. flavus* and the 6 isolates of *A. parasiticus* giving positive results using TLC had succeeded in giving rise illumination upon examining their agar media under UV light. In addition, some more isolates (4 & 2 of *A. flavus* and *A. parasiticus*, respectively) which failed to give positivity using TLC, exhibited illumination under UV light. These findings lay more or less in agreement with those recognized by Hara et al. (1974).

It is clear from the results presented in table 4, explaining the types of fragmented aflatoxins detected by TLC, that aflatoxin B_1 constituted 45.5% and 50% of the total aflatoxins produced by the tested strains of *A. flavus* and *A. parasiticus*, respectively. Aflatoxins B_2, G_2 and G_1 came less frequently in decreasing order. Bullerman (1979) stated that aflatoxins B_1, B_2, G_1 and G_2 are the major types produced by aspergilli, however B_1 and G_2 are usually synthesized in larger amounts. Concerning aflatoxin M_1 is the metabolite of B_1, our findings here came parallel with those reported by Abouzeid et al. (1996) on evaluating the existing mycotoxins in Roume and Kareish cheeses. Such aflatoxins may be produced in various types of cheese if the aspergilli in concern had the chance to grow in the product during storage at favorable temperature and humidity (Kiermeyer, 1971).

From the above, it is very disappointing to say that Egyptian cheeses constitute a hazardous source of mycotoxins due to its continence of various types of mould contaminants having the ability of their production. Also, it is concluded from this work, that fluorescence technique of the agar medium has the ability to screen the production of aflatoxins by aspergilli. Indeed, it could be rely upon more beneficially if it is used in the exclusion and elimination of negative aflatoxigenic ones.

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Table 1: Statistical analytical results of mould count (cfu/g) in examined cheese samples

Type of cheese	No. of samples	Positive samples		Mould count (cfu/g cheese)		
		No.	%	Min.	Max.	Average
Kariesh	30	22	73.3	30	4.6×10^4	3.9×10^3
Damietta	30	13	43.3	20	7.4×10^4	5.1×10^3
Ras	30	24	80.0	25	2.9×10^5	7.2×10^4
Processed	30	15	50.0	45	6.5×10^3	2.3×10^2

Table 2: Incidence of mould species isolated from the examined cheeses

Mould species	Kariesh		Damietta		Ras		Processed	
	No.	%	No.	%	No.	%	No.	%
Aspergilli	16	53.3	9	30.0	18	60.0	6	20.0
A.flavus	7	23.3	5	16.7	9	30.0	3	10.0
A. parasiticus	4	13.3	4	13.3	6	20.0	1	3.3
A.niger	4	13.3	-	-	4	13.3	2	6.7
A. ochraceous	5	16.7	2	6.7	3	10.0	2	6.7
A. terrus	2	6.7	-	-	1	3.3	-	-
Penicillia	7	23.3	4	13.3	9	30.0	11	36.7
P.citrum	1	3.3	1	3.3	4	13.3	2	6.7
P. expansum	1	3.3	1	3.3	2	6.7	3	10.0
P. verricosum	3	10.0	2	6.7	3	10.0	6	20.0
P. rubrum	1	3.3	-	-	1	3.3	2	6.7
P. diversum	2	6.7	2	6.7	2	6.7	-	-
Mucor spp.	2	6.7	-	-	3	10.0	1	3.3
Geotrichum spp.	3	10.0	2	6.7	3	10.0	2	6.7
Cladosporum spp.	2	6.7	1	3.3	2	6.7	1	3.3

Table 3: Incidence of aflatoxins producing moulds within the isolated *A. flavus* and *A. parasiticus* strains using both the fluorescence and TLC methods.

Aspergillus Species	No. of Tested Isolates	Positive aflatoxigenic strains			
		Fluorescence method		TLC method	
		No.	%	No.	%
A. flavus	24	15	62.5	11	45.8
A. parasiticus	15	8	53.3	6	40.0

Table 4: Types of aflatoxins produced by the isolated toxigenic aspergilli as detected by the TLC

Aspergillus Species	No. of Tested isolates	Types of aflatoxins							
		B_1		B_2		G_1		G_2	
		No.	%	No.	%	No.	%	No.	%
A. flavus	11	5	45.5	3	27.3	2	18.2	1	9.1
A. parasiticus	6	3	50.0	1	16.7	-	-	2	33.3