Dept. of Food Hygiene,
Animal Health Research Institute, Assiut, Regional Laboratory

DETECTION OF AFLATOXINS RELEASED BY SOME ASPERGILLUS SPECIES ISOLATED FROM PROCESSED CHEESE TOGETHER WITH SOME AEROBIC AND ANAEROBIC SPORE FORMER ORGANISMS

(With 4 Tables)

By

NAHED M. WAHBA and EMAN KORASHY AHMED

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الكشف عن السموم الفطرية المفرزة من بعض أنواع الأسبرچيلس المعزولة من الجبن المطبوخ وبعض الميكروبات المتحوصلة الهوائية واللاهوائية

ناهد محمد وهبه ، إيمان قرشى أحمد

فى السنوات الأخيرة زاد الإقبال على استهلاك الجبن المطبوخ وذلك لقناعة المستهلك بسلامة هذا النوع من الجبن نظراً لطريقة تصنيعه وتعرضه للحرارة أثناء الطبخ. لذا هدفت الدراسة إلى معرفة مدى تلوث الجبن المطبوخ بالفطريات وخصوصاً المفرزة منها لسموم الأفلاتوكسين وكذلك تواجد الميكروبات المتحوصلة الهوائية واللاهوائية. لذا تم تحليل 120 عينة من الجبن المطبوخ وأظهرت النتائج أن 18 (15%) من العينات كانت تحتوى على فطريات وخمائر وكان العدد الكلى لها 2.7×10^4 لكل جرام وأعلى نسبة بين العينات الموجبة (55.6%) كانت لفطر الأسبرجلس فلافس والذى أثبتت النتائج أن 15% منه كانت مفرزة لجميع أنواع الأفلاتوكسين. أما بالنسبة للميكوتوكسين فقد تبين أن 5 عزلات من بين 20 عزلة كانت مفرزة للميكوتوكسين والتى تسبب في موت أكثر من 50% من اليرقات المختبرة . و تناولت الدراسة أيضاً تواجد الميكروبات المتحوصلة الهوائية واللاهوائية وقد أمكن عزل الباسيلس سيرس بنسبة 14.2% بمتوسط عددى 7×10^2 ميكروب / جرام كما أمكن عزل كل من الباسيلس ماسيرنس ، الباسيلس ليشنيفورم والباسيلس ميجاتيرم بالنسب الآتية 6.7 ، 16.7 ، 11.7% على التوالي، وكذلك الكلوستردينم بيرفنجنز بنسبة 13.3% من العينات المفحوصة. ولقد خلصت الدراسة إلى ضرورة إتباع بعض الخطوات لإنتاج جبن مطبوخ سليم وآمن تتلخص في الخطوة الأولى: منع تلوث غذاء الحيوان بالفطريات حتى نمنع إفراز الأفلاتوكسين ومنع تكاثرها داخل المزرعة. الخطوة الثانية: تشمل بعض الاحتياطات اللازمة لمنع نمو وتكاثر الميكروبات في الجبن وذلك بالتبريد السليم فى كل مرحلة من مراحل التصنيع وحتى يصل إلى المستهلك.

SUMMARY

Processed cheese has an excellent history of safety; however, it is difficult to eliminate completely fungi and bacterial growth, activity and toxin production that threaten consumer's health. Methods: 120 processed cheese samples were analyzed for determination of total fungi and yeast count, as well as identification of the isolated fungi. Toxicity and aflatoxin produced by the isolated *Asp. flavus* using thin layer chromatographic technique. Bacillus species and *C. perfringens* count as spore former organisms. Results: Fungi and yeasts were present in 18 (15%) of processed cheese samples with total count of $2.7x10^4$/g. *Asp. flavus* was the first prevalent species (55.6%) of the positive samples. 5 (25%) out of 20 isolates of *Asp. flavus* proved to produce mycotoxins, three of them were able to produce all types of aflatoxins. Mycotoxins producing isolates cause more than 50% mortality of the larvae tested. Some aerobic and anaerobic sporeformers were isolated and counted. *B. cereus* was isolated from 17 (14.2%) of processed cheese samples with an average count of $7x10^2$/g. *B. macerans, B. lechniformis* and *B. megaterium* were detected in 8 (6.7%), 20 (16.7%) and 14 (11.7%) of samples, respectively. Moreover, *C. perfringens* was present in 16 (13.3%) of samples. Conclusion: Two important points must be regarded to safe production of processed cheese; first one: is the measures to minimize the presence of spores. The second is to prevent spore germination and vegetative proliferation by adequate cooling during all steps of production till dispensation to the consumers.

Key words: *Processed cheese, Aspergillus ssp., aflatoxins*

INTRODUCTION

Processed cheese has an excellent history of safety. Production and consumption of processed cheese increased steadily. Today, large tons of natural cheeses are converted to processed cheese, the global production of processed cheese products is estimated at ~1.5-1.8 million ton/year (Guinee, 2003). Heat processing, packaging and cheese composition normally limit bacterial growth however, it is difficult to eliminate completely bacterial growth and activity in this type of cheese (Hamed *et al*., 1997). Airborne mold spores tend to grow on cheese where pockets of air exist between the packaging material and cheese surface (Hocking and Faedo, 1992).

Aflatoxins (Afs) are hepatotoxic, mutagenic, carcinogenic and immuno suppressive toxins (Bennett and Klich, 2003 and Honikel, 2003). They are secondary metabolites produced by many strains of *Aspergillus flavus* and *Asp. parasiticus*. The major aflatoxins of concern are designated Af B_1, B_2, G_1, G_2 and M_1. Af B_1 is predominant in amount and toxicity (Mountney and Gould, 1988). Outbreaks of mycotoxicosis from Af B_1 have been reported (Peraica *et al*., 1999). Cattle consume Af B_1 in feedstuff and transform it into Af M_1 so, milk and milk products can serve as indirect source of aflatoxins (Kisza and Domagala, 1994). Af B_1 and M_1 are highly toxic compound and its occurrence poses a threat to the health of consumers specially young children (Pierides *et al*., 2000).

Furthermore, aflatoxins are relatively stable compounds, not destroyed by processing and may even be concentrated. Their thermal stability disqualifies pasteurization and ultra pasteurization as methods of control (Carvajal *et al*., 2003 and Honikel, 2003).

Endo spore forming species of Bacillus and Clostridia are of great concern to the dairy industry causing both food spoilage and food poisoning. *B. cereus* and related species cause problems to the dairy industry by deteriorating the product (Eneroth *et al*., 2001) and by endangering people's health up consuming contaminated food (Ghelardi *et al*., 2002). *B. cereus* is considered to be the most important species however, more recently attention has been drawn to other species of bacillus that may occur in milk products more often and in higher concentrations than *B. cereus* (Mansour *et al*., 1999).

Likewise, *C. perfringens* is a classical agent of foodborne disease characterized by mildness and self limiting nature (Sanz *et al*., 2002). *C. perfringens* type A is considered the third leading cause of foodborne disease in the USA (Bos *et al*., 2005). Species of clostridia producing butyric acid like *C. butyricum*, C. perfringens and *C. tertium* cause sever defects in cheese as a consequence of late blowing or butyric blowing (Galle le Bourhis *et al*., 2005).

Therefore, and due to the dangerous role of molds in cheese, prevalence and count of mold are considered as standard test for checking the general sanitary measures adopted, together with toxin production and toxicity of *Asp. flavus* isolates. Moreover, the incidence and count of Bacillus species and *C. perfringens* are essential.

MATERIALS and METHODS

A total of 120 samples of processed cheese were collected and subjected to the following:

1- Preparation of the samples according to APHA (1992).

2- Enumeration and Isolation of molds and yeasts were done as described by Oxoid (1990).

3- Identification of isolated fungi was done in the Mycological Center, Fac. of Science, Assiut Univ.

4- Cultivation and extraction of the fungal toxins:

Asp. flavus isolates were collected and cultivated on Czapek's liquid medium fortified by 0.2% yeast extract and 1% peptone, incubated at 28°C for 10 days. The content of each flask (medium and mycelium) were homogenized for 5 min. in high speed blender (1600 rpm) with 100 ml chloroform. The extract procedure was repeated 3 times, washed with equal volume of dist. water, dried over anhydrous sodium sulfate, filtered then, concentrated under vacuum to near dryness and diluted to 1 ml with chloroform.

5- Thin-layer chromatographic separation of mycotoxins:

Thin-layer chromatographic technique was used for qualitative identification of mycotoxins by comparison with appropriate reference standards (Gimeno, 1979 and Van Egmond *et al*., 1980).

6- Bioassay method for mycotoxins:

The immature brine shrimp (Artemia salina L.) was used for detection of aflatoxins and for other mycotoxins toxicity as described by Scott *et al*. (1980).

7- Enumeration, isolation and identification of Bacillus spp. using Mannitol egg yolk polymyxin agar (MYP) according to Lancette and Harmon (1980) and Cowan and Steel (1974).

8- Enumeration of *C. Perfringens* using lactose sulphite broth (Beerens *et al*., 1982) by applying most probable number (MPN) technique.

RESULTS

The results were manifested and tabulated in Tables (1-4).

Table 1: Incidence and count of different molds and yeasts isolated from processed cheese samples.

Mycoflora	No. of +ve samples	Frequency %	Total	Range
Genera and species	No/18			
Asp. flavipes	1	5.6	100	100
Asp. flavus	10	55.6	3800	100-1400
Asp. fumigatus	1	5.6	2000	2000
Asp. niger	8	44.4	1500	100-300
Asp. ochraceus	1	5.6	100	100
Asp. terrus	1	5.6	6000	6000
Asp. versicolor	1	5.6	100	100
Cochliobolus lunatus	2	11.1	300	100-200
Cochliobolus spicifer	1	5.6	100	100
Emericella quadrilineata	7	38.9	1800	100-500
Fusarium verticillioides	4	22.2	500	100-200
Mucor hiemalis	1	5.6	200	200
Paecilomyces roseolus	1	5.6	100	100
Penicillium citrinum	1	5.6	100	100
Penicillium funiculocum	3	16.6	300	100
Penicillium purpurogenum	2	11.1	200	100
Rhizopus stolonifer	2	11.1	200	100
Yeasts	5	27.7	9700	100-5000
Total mold and yeast count	18/120	15	$2.7x10^4$	100-6000

Table 2: Aflatoxin production and toxicity of *Asp. flavus* isolated from processed cheese samples.

No. of tested isolates	*Asp. flavus* isolates producing					Toxicity test			
	Mycotoxins		Aflatoxins			A		B	
	No.	%	No.	%	Types	No.	%	No.	%
20	5	25	3	15	B_1, B_2, G_1, G_2 and M_1	5	25	15	75

A: indicates more than 50% mortality of larvae tested.
B: indicates less than 50% mortality of larvae tested.

Table 3: Incidence and count of some aerobic spore former organisms in processed cheese samples.

Isolated organisms	Positive samples		Count /g		
	No./120	%	Min.	Max.	Average
B. cereus	17	14.2	$1x10^2$	$3.8x10^4$	$7x10^2$
B. macerans	8	6.7	$1x10^2$	$5x10^3$	$2x10^2$
B. lechniformis	20	16.7	$1x10^2$	$7x10^3$	$3x10^2$
B. megaterium	14	11.7	$2x10^2$	$7x10^3$	$3x10^2$

Table 4: Incidence, count and frequency distribution of *C. perfringens* in processed cheese samples.

No. of samples examined	Positive samples		MPN/g		
	No.	%	3-20	20-70	70-1100
120	16	13.3	15	-	1

DISCUSSION

Consumer demand for processed food challenges the food industry to produce tasty-nutritious and microbially safe products (Leistner and Gorris, 1995). Mycobiota growing in food is often beneficial for the ripening and development of specific flavor characteristics of the product, but it can be harmful due to the production of mycotoxins (Federico *et al*., 2002).

Mold and yeasts were present in 18 (15%) of processed cheese samples with total count of $2.7x10^4$ (Table 1). Higher incidence and count were reported by Wafy (2006) and Amin (2007). 100% incidence was detected by Ismail and Sabreen (2001) and Sabreen and Zaky (2001). On the other hand, no molds could be isolated from processed cheese examined by Taniwaki and Dender (1992). Processed cheese may serve as a substrate to *Asp. flavus* and other mycotoxins producing fungi (Sabreen and Zaky, 2001 and Amin, 2007).

Asp. flavus was the first prevalent species encountered in processed cheese samples. It was found in 10 (55.6%) of the positive samples in counts ranged from 100-1400 cells/g, other species were isolated in different percentages and counts (Table 1). The considerable high incidence and count of *Asp. flavus* is of great importance for its production of Aflatoxins. Several researches detected aflatoxins in

processed cheese (Sabreen and Zaky, 2001; Sarimehmetoglu *et al*., 2004 and Yaroglu *et al*., 2005). Af B_1 and M_1 are highly toxic compound and its occurrence poses a threat to the health of consumers specially young children (Pierides *et al*., 2000).

In the present study, 5(25%) out of 20 isolates of *Asp. flavus* proved to produce mycotoxins, three of them were able to produce all types of aflatoxins (B_1, B_2, G_1, G_2 and M_1). All mycotoxins producing isolates (5 isolates) cause more than 50% mortality of the larvae tested (Table 2). The mycotoxin production and toxicity by *Asp. flavus* wes previously studied by El-Maraghy and Zohri (1988) and Abdel-Malek *et al*. (1993).

Because mycotoxins are natural contaminants of food and their exposure is more likely to occur in world where poor methods of food handling and storage are common, their formation is unavoidable and methods of controlling are usually preventive.

Aerobic spore formers like Bacillus species naturally existed in numerous foods owing to their wide distribution in the environment. Growth of *B. cereus* often limits the shelf life of dairy products kept at refrigerator temperatures, if conditions are right, they multiply rapidly and produce sufficient toxins to induce symptoms of food poisoning (Borge *et al*., 2001 and Vissers *et al.*, 2007).

B. cereus was previously isolated from processed cheese by Wahba (1997); Sabry (2001) and Abdel-Hameed (2004). In this work, *B. cereus* was isolated from 17 (14.2%) of processed cheese samples with counts ranged from $1x10^2$ to $3.8x10^4$, with the average count was $7x10^2$ organisms/g (Table 3). Concentration of $<10^3$/g may considered innocuous since the minimal level required to cause illness has been estimated to be $>10^5$/g (ICMSF, 1996).

B. macerans, B. lechniformis and *B. megaterium* were detected in 8 (6.7%), 20 (16.7%) and 14 (11.7%) of samples, respectively (Table 3). Although some foodborne gastroenteritis incidents attributed to *B. lechniformis* have been reported (Turnbull, 1997 and Salkinoja-Salonen *et al*., 1999), *B. lechniformis* secreted protease enzyme which produce milk curds and may be useful as a new source of milk coagulants for cheese making industry (Ageitos *et al*., 2007).

The clostridium species present in cheese originate from milk which is contaminated by these spores during milking, even >0.2 clostridium spores/ml in raw milk can affect the cheese during processing (Kalzendorf, 1995).

C. perfringes was present in 16 (13.3%) of processed cheese samples. Most of samples lines within the range of 3-20 cfu/g (Table 4). Lower incidences of *C. perfringens* and other anaerobic organisms were observed by Wahba (1997) and Amin (2007).

In most instances the actual cause of poisoning of *C. perfringens* is temperature abuse, small numbers of organisms are present, multiply to food poisoning levels during cool down and storage (Crouch and Golden, 2005).

So, two important points must be regarded to safe production of processed cheese, first one is the measures to minimize the presence of spores including low initial contamination levels in the feed and preventing their growth in the farm environment. The second is emphasizing the requirement for precautions that prevent spore germination and vegetative proliferation by adequate cooling during all steps of production till dispensation to the consumers.

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