قسم الرقابة الصحية على الاغذية
كلية الطب البيطري ـ جامعة أسيوط
رئيس القسم : أ٠د/ توفيق البسيوني

دراسات ميكولوجية على بعض التوابل التي تدخل في صناعة منتجات اللحوم

حسني عبداللطيف

بالفحص الميكولوجي لعدد ٥٠ عينة من التوابل التي تستخدم في صناعة منتجات اللحوم المصرية • وجد أن العدد الكلي للفطريات في العينات المفحوصة تتراوح بين ٢٠٠ الى ٣٫٤ مليون/جرام وأن الفلفل الابيض والاسود والاحمر وكذلك الشطة تحتوي على أعداد كبيرة من الفطريات •

وقد تم عزل ٥٠٧ نوع من جنس الاسبرجلس فلافس وتصنيفه ودراسة قدرته على افراز سموم الافلاتوكسين • كما ثبت أن ١٥١ نوع مفرزين للسموم الفطرية المختلفة B_1, B_2, G_1, G_2 وأن ٧٩٪ من الفطريات السامة أفرزت سم الافلاتوكسين B_1 و ١٩٫٨٪ افرزت B_2 ٢١٫٨٪ أفرزت G_1 و٦٪ أفرزت G_2 •

وقد اتضح من البحث أن التوابل التي تستخدم في صناعة منتجات اللحوم بوضعها الحالي بدون معاملات تعتبر مصدرا جيدا لتلوث منتجات اللحوم بالفطريات وخصوصا المفرزة للسموم الفطرية التي بالتالي تضر بصحة المستهلك بما لها من تأثير سرطاني وخصوصا على الكبد ويجب على المصنعين لمنتجات اللحوم من ازالة هذا التلوث بالطرق المختلفة وخصوصا التعقيم بالاشعاع (أشعة جاما) •

Dept. of Food Hygiene,
Faculty of Vet. Med., Assiut University,
Head of Dept. Prof. Dr. T. El-Bassiony.

MYCOLOGICAL STUDIES ON SOME SELECTED SPICES WITH SPECIAL REFERENCES TO AFLATOXIN PRODUCING ASPERGILLUS FLAVUS SPECIES

(With 5 Tables)

By
H. ABD EL-RAHMAN
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SUMMARY

The qualitative and quantitative estimation of fungal flora of some selected meat products spices included, white pepper, black pepper, red pepper, capsicum and cummin were studied with special references to Aspergillus flavus group. The average mould count in the examined samples of spices ranged from 200 to 3.4 Million mould spores/g. White pepper and black pepper were heavy loaded with mould spores. The isolated Aspergillus flavus group were screened for their toxigenic properties. A total of 507 Aspergillus flavus isolates were tested, 90(48.9%) isolates were confirmed to be aflatoxins producers. 79.5% of the toxic isolates produced aflatoxin B_1, 19.8% produced B_2, 21.8% produced G_1 and only 6.0% produced G_2. The significance importance of the isolates and preventive measures were discussed.

INTRODUCTION

The occasional contamination of meat products by a wide range of mould species, reflects mostly an important source of mould contamination, arising from the use of natural untreated spices in manufacturing of meat products. The mycological investigation concerning the quantitative estimation of mould of different types of spices received considerable attention by several investigators (YESAIR and WILLIAMS, 1942; WESTERDIJK, 1949; CORETTI, 1957; POHJA, 1957; ESCHMANN, 1965; CHRISTENSEN, 1967 and SENSER, 1967.

HADLOK (1970) studies the qualitative and quantitative estimation of the fungal flora of 103 samples of spices (Cummin, marjoran, capsicum, black pepper, white pepper, mustard, onion powder and thyme) and concluded that 70% of the examined samples contained up to 5×10^4 mould/g. Aspergillus species lied with 70%, Penicillium species 20%, and other mould species 10%, while Aspergillus flavus group with 10% and Aspergillus glaucus group with 25% from the total Aspergillus.

Aspergillus species are known to occur naturally in levels sufficient to be regarded as significant hazards to animals and human health (DAVIS, 1981). Most records have pointed out to members of Aspergillus flavus group, particularly A.flavus and A.parasiticus as a source of aflatoxins (RAPER, et al. 1965).

DIENER and DAVIS (1966) screened various isolates of A.flavus for aflatoxin-production and found that 80% of the isolates produced aflatoxin, 90% of which produced primarly aflatoxin

B_1 and 10% produced both aflatoxin B_1 and G_1. HADLOK (1970) screened 153 isolates of A.flavus isolated from black pepper and found that 30% of the isolates were aflatoxin producers, while the four isolates of A.flavus and A.flavus var.columnaris which isolated from meat products, were not aflatoxin producing species. Federal RIGISTER (1974) reported that the presence of aflatoxin producing mould on a food does not necessarily imply the presence of the aflatoxin, conversely, the absence of obvious growth of an aflatoxin-producing mould does not indicate more or less the absence of the toxin, since aflatoxin may be produced when little mould growth is evident. Furthermore aflatoxins may remain in a food product after processing. In concern, adequate research has not been directed to determine the frequency occurrence of mould and their aflatoxin-producing species in meat products additives in Egypt, therefore the present study is carried out to determine the qualitative and quantitative estimation of the fungal flora of some selectd meat products spices including screening test for determining the aflatoxin-producing isolates, specially species of Aspergillus flavus group.

MATERIAL and METHODS

A total of fifty samples of untreated spices, included five of the most widely used in manufacturing of meat products each 10 samples of cumin, black pepper, white pepper, red pepper and capsicum, were choosen for this investigation which include:

1) Enumeration of total mould count:

The quantitative estimation of the total mould count/g of the examined samples of above mentioned spices was carried out according to RAPER, et al. (1965) and HADLOK (1970): 5 gm of finelly grinded spice, 74 ml of sterile physiological saline and one ml 1% of sterile sodium lauryl sulfonate were throughly homogenised for 30 second in waring blender at 12000 r.p.m. The mixture was allowed to stand steady for 2 minutes, then 20 ml of 10% carboxymethyl cellulose (CMC 35-37%) followed by second homogenisation for one minute at 12000 r.p.m. to obtain final dilution of 1/20, from which 10-Fold dilutions were carried out in physiological saline with 2% C.M.C.Plating were carried out by using Acidified malt extract agar (pH 4.5) for enumeration of the total mould count, and acidified Czapek-Dox -agar with 17% sacchrose for enumeration the osmophilic mould specially Aspergillus glaucus group. The inoculated plates were incubated at 25C° for 7 days, during which the mould colonies exhibiting star shape were transferred onto malt extract slope agar with 3% sacchrose and kept for the qualitative estimation. The identification of mould isolates were carried out according to RAPER, et al. (1965), SAMSON (1979) ARX (1967) and BARNETT and HUNTER (1972).

2) Screening of the aflatoxin-producing Aspergillus flavus species:

2.1 - Cultivation:

A total of 507 isolates of Aspergillus flavus species, included A.flavus (184); A.flavus var. columnaris (190); A.oryzae (78) and A.parasiticus (55), were inoculated at the center of solidified agar medium in glass petri-dishes (Fluorescence Agar medium according to HARA, et al. 1974) and incubated at 25 C°

2.2 - Observation of Fluorescence:

The plates were examined under UV (366 nm) illumination starting from the seventh day of incubation up to the tenth day for the detection of the blue fluorescence in the agar surrounding colonies.

2.3 - Cultivation and extraction of Aflatoxins:

The Aspergillus flavus species which illuminate blue fluorescence in the solid agar medium were inoculated in rice medium (SHOTWELL, et al. 1966) for 5 days at 25 C°. At the end of the incubation time 25 ml of chloroform was added and the mixture was throughly homogenised for one minute in Ultralurax apparatus, then the mixture was centrifuged (3000 r.p.m.) for 10 minutes where the chloroform layer was decanted. The chloroform extraction was repeated only once. One ml ethanol, 3 gm copper-(111)-hydroxidcarbonate and from 5-10 gm anhydrous sodium sulphate wer added to th chloroform extract, mixed well and filtered. The filterate was then evaporated in rotatory vaccum evaporator.

2.4 - Determination and confirmation of Aflatoxin:

(SCHULLER and EGMOND, 1981)

The concentrated extract was spotted onto activated thin layer chromotography plates coated with silica gel of 0.25 mm thickness. The plates were then developed firstly in diethyl ether until the solvent path length of 15 cm from the base line has been obtained. The plates were then air dried at room temperature for five minutes. The chromatoplates were re-developed in a homogenous solution of chloroform : methanol (97 : 3 v/v) until the solvent path length of 12 cm from the base line has been obtained. Developed plates were dried for five minutes in dark and examined under UV Lamp (366 nm) illumination.

The toxins were idenitified according to their emission of either/or blue or green fluorescence coloration, R_f -Value (Retention factor) and with the use of matching authentic referance aflatoxin standards, which were soptted side by side with the extract on the chromatoplates. Moreover confirmation of aflatoxin were carried out by using Trifluoroacetic acid (TFA) according to PRZYBYLSKI (1975) and VERHULSDONK, et al. (1977).

RESULTS

The results were recorded intables 1 to 5.

DISCUSSION

The qualitative and quantitative estimation of mould in the examined samples of Cummin, Black pepper, White pepper, Red pepper and Capsicum as shown in Table (1) revealed that, the average mould count/g were 10^4, $3x10^5$, $2x10^6$, $3x10^4$ and $4x10^4$ respectively. The highest counts were observed in white and black pepper, the range were $2x10^3$ to $3x10^6$ and 10^3 to $2x10^6$ respectively. These findings are nearly similar to those reported by YESAIR and WILLIAMS (1942); CHISTENSEN, et al. (1967); CORETTI (1957) and HADLOK (1970).

The distribution of mould genera (Table 2) showed that the genus Aspergillus lied with the highest count and percentage among the examined types of spices. The average count in cummin, $5x10^5$ white pepper, 10^4 black pepper, red pepper 10^4 and capsicum wer $5x10^3$ (44.5%), $8x10^5$ (35.2%), 10^5 (45.0%), 10^4 (38.9%) and $2x10^4$ (64.2%) respectively. These findings are nearly similar to those reported by HADLOK (1970).

The Aspergillus flavus group lied with the highest count in white pepper, red pepper and black pepper with the following count and percentages; $5x10^5$ (22.0%), $5x10^3$ (17.2%),

and $4x10^4$ (15.0%) respectively. These results are nearly similar to those reported by HADLOK (1970).

Aspergillus glaucus group could be detectd with the highest count in black pepper, cummin, white pepper with the following averages of $8x10^4$ (30.0%), $3x10^3$ (21.8%) and $7x10^3$ (21.7%) respectively, nearly similar results were recorded by HADLOK (1970).

Penicillium species lied with the highest count and percentages in white pepper and capsicum with $7x10^5$ (28.7%) and $4x10^2$ (11.7%) respectively, while in cummin, black pepper and red pepper the values were less than 5%, these findings are similar to those reported by HADLOK (1970).

The other mould species including A.niger, A.fumigatus, A.terreus, Mucor, Absidia, Rhizopus, Cladosporium, Paecilomyces, Trichothecium were calculated togther, the highest count and percentage were obtained in capsicum, red pepper, cummin, and white pepper with $7x10^3$ (20.6%), $6x10^3$ (19.8%), $2x10^3$ (15.9%) and $2x10^4$ (0.9%) respectively.

The distribution of identified A.flavus group (Table 3) showed that, the white pepper and red pepper were heavy loaded with Aspergillus flavus and lied with the following average and percentages $2x10^5$ (40.0%), $2x10^4$ (37.5%) and $2x10^3$ (27.7%) respectively. These results are nearly similar to those reported by CHRISTENSEN, et al. (1967) and HADLOK (1970). A.flavus var columnaris, A.oryzae and A.parasiticus, could be detected with highest count in white pepper, black pepper and red pepper with the following count/g, 10^3, $2x10^4$ and $3x10^3$; 10^5, $5x10^3$ and 10^5; $8x10^5$, $3x10^3$ and $4x10^2$ respectively.

Aspergillus chevalieri lied with the highest count in black pepper, and white pepper with count of $2x10^4$ and $6x10^3$ respectively, while in cummin, red pepper and capsicum the counts were nearly 10^2. Aspergillus amstelodami was the more predominant Aspergillus species within the Aspergillus glaucus group in the examined types of spices, specialy in capsicum and white pepper with count of $3x10^2$ (43.3%) and $5x10^5$ (97.0%) respectively. Aspergillus repens lied with 10^2 (16.0%), $4x10^2$ (14.2%) and 10^4 (12.3%) in capsicum, cummin and red pepper respectively while Aspergillus ruber lied with the lowest percentage within the Aspergillus glaucus group (Table 3).

Screening of the Aflatoxin-producing Aspergillus flavus species:

Aflatoxins are secondary metabolites produced by strains of Aspergillus flavus Link, A.parasiticus-Speare and other Aspergillus species which have been shown to be both toxic and carcinogenic in test animals (BULLERMAN, 1974). It has been reported that these toxins may also be involved in the etiology of human liver cancer in certain parts of the world (DENIZEL and KOSKER, 1972).

The results obtained in this study (Table 4 & 5) revealed that 151(29.8%) out of 507 isolates of Aspergillus flavus group were aflatoxin-producing species. A total of 184 Aspergillus flavus Link isolates were screened for their toxigenic properties, 90 isolates were found to be aflatoxin producers with percentage of 48.9%. All the toxic isolates provided to produced B_1 while 10 isolates of them were found to produce both B_1 and B_2 with percentage of 11.1%. These findings were in agreement with those reported by TABER and SCHROEDER (1969); BOLLER and SCHROEDER (1966) but HADLOK (1970) found that B_1 and G_1 could be detected in 4(9%) of Aspergillus flavus Link isolates.

Aspergillus flavus var columnaris lied with 33(17.4%) out of 190 isolates as toxic species, and aflatoxin B_2 was predominant than B_1. Also aflatoxin G_1 and G_2 found to be produced

by 9(27.3%) and 2(6.1%) of them. Aspergillus oryzae lied with 15(19.2%) out of 78 isolates as toxic species and aflatoxin G_1 was the predominant toxin, but B_1, B_2 could be also detectd. Aspergillus parasiticus lied with 13(23.6%) out of 55 isolates as toxic, both toxins B_1 and G_1 were detected in all isolates. These findings were in agrement with those reported by HESSELTINE, et al. (1968) and MILNE, et al. (1968).

From the results obtained in this study, it is achieved that, meat products were contaminated with toxigenic Aspergillus flavus species, when the natural untreated spices were used in the processing of such meat products. It remains uncertain whether any of non-producing species of Aspergillus flavus group may able to produce aflatoxins under other condition when different substrates or enviroment or both are used. COLE (1976) stated that all Aspergillus species should be considered hazards to humen and animal health until proven otherwise. It appears quite necessary for the sake of the meat processors that they convince themselves regularly by mycological checking. Moreover sterilization of such spices must be done by using appropriate methods.

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Table(I):Statistical analytical results of total mould count/g of examind spices.

	Cummin	Black pepper	White pepper	Red pepper	Capsicum
Minimum	$2xIO^2$	IO^3	$2xIO^3$	IO^2	$3xIO^2$
Maximum	6xIO	$2xIO^6$	$3xIO^6$	$3xIO^4$	$5xIO^4$
Mean	IO^4	$3xIO^5$	$2xIO^6$	$3xIO^4$	$3xIO^4$

Table(2): Averages and percentages of the isolated Aspergillus and Penicillium in the examined spices

Type of spices	Aspergillus		A.flavus		A.glaucus		Penicillium		Other mould	
	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%
Cummin	$5x10^3$	44.5	$2x10^3$	14.3	$5x10^3$	21.8	$4x10^2$	3.5	$2x10^3$	15.9
Black pepper	10^5	45.0	$4x10^4$	15.0	$8x10^4$	30.0	10^4	4.1	$2x10^4$	5.9
White pepper	$8x10^5$	35.2	$5x10^5$	22.0	$3x10^5$	13.2	$7x10^5$	28.7	$2x10^4$	0.9
Red Pepper	10^4	38.9	$5x10^3$	17.2	$7x10^3$	21.7	$8x10^2$	2.4	$6x10^3$	19.8
Capsicum	$2x10^4$	64.2	$5x10^2$	1.4	$8x10^2$	2.1	$4x10^2$	11.7	$7x10^3$	20.6

Table(3): Averages and percentages of the identified Aspergillus flavus and glaucus groups.

Type of spices	Aspergillus flavus group								Aspergillus glaucus group							
	A.flavus		A.flavus var columnaris		A.oryzae		A.parasiticus		A.chevaliere		A.amstelodami		A.repens		A.ruber	
	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%
Cummin	$5x10^2$	30.0	$6x10^2$	36.5	$2x10^2$	30.6	10	2.9	$6x10^2$	24.2	10^3	54.6	$4x10^2$	14.2	$2x10^2$	6.9
Black pepper	$2x10^4$	37.5	$2x10^4$	42.5	$5x10^3$	12.5	$3x10^3$	7.5	$2x10^4$	25.0	$5x10^5$	62.5	10^4	12.5	$2x10^2$	0.3
White pepper	$2x10^5$	40.0	10^5	20.0	10^5	24.0	$8x10^4$	16.0	$6x10^3$	2.0	$3x10^5$	97.0	$2x10^3$	0.7	10^3	0.3
Red pepper	$2x10^3$	27.7	$3x10^3$	46.1	10^3	18.5	$4x10^2$	7.7	$6x10^2$	8.8	$6x10^3$	85.0	$4x10^2$	5.9	20	0.3
Capsicum	$2x10^2$	39.6	$2x10^2$	41.6	60	12.5	30	6.3	$2x10^2$	29.3	$3x10^2$	43.3	10^2	16.0	10^2	11.4

Table(4):Number and percentage of toxigenic Aspergillus flavus group in some selected spices

Type of Spices	A.flavus Link			A.flavus var columnaris			A.oryzae			A.parasiticus Spear		
	T.NO.	NO.+ve	%	T.NO.	NO.+ve	%	T.NO.	NO.+ve	%	T.NO.	NO.+ve	%
Cummin	II	2	I8.2	9	2	22.2	II	2	I8.2	2	-	-
Black pepper	52	20	38.5	55	8	I4.5	I9	4	2I.I	I5	4	30.8
White pepper	62	33	53.2	78	I3	I6.7	2I	2	9.5	I6	3	I8.8
Red pepper	32	2I	65.6	I6	I	6.3	9	I	II.I	I	-	-
Capsicum	27	I4	5I.9	32	9	28.I	I8	6	33.3	23	6	26.I

Table(5) : Statistical analytical results of numbers,types and percentages of Aflatoxin-producing Aspergillus flavus group in some selected spices

Aspergillus flavus group	Total NO. of isolates	Aflatoxin +ve isolates		Types of aflatoxins							
				B_1		B_2		G_1		G_2	
		NO.	%	NO.	%	NO.	%	NO.	%	NO	%
A.flavus Link	I84	90	48.9	90	I00	I0	II.I	-	-	-	-
A.flavus var columnaris	I90	33	I7.4	I4	42.4	I9	58.0	9	27.3	2	6.I
A.oryzae	78	I5	I9.2	3	20.0	I	6.7	II	73.3	7	46.7
A.parasiticus Spear	55	I3	23.6	I3	I00.0	-	-	I3	I00.0	-	-