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Screening of Aflatoxigenicity of *Aspergillus flavus* Strains in Artificially Contaminated Yoghurt Samples

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Abstract: The aim of the present study was to experimentally investigate the toxicity of *Aspergillus flavus* (*A. flavus*) strains in yoghurt. The inoculated strains were isolated from cheese and yoghurt. The inoculated conidial count was adjusted at 2-5 x 10^3/ ml. Results revealed that all tested strains were toxigenic and produced aflatoxins with different concentrations as measured by HPLC. The highest toxin concentration was Aflatoxin B_1 (134.77 ppm). According to these findings it could considered that *A. flavus* at different concentrations was able to producing aflatoxins if yoghurt kept over 5°C. Therefore, yoghurt must be *A. flavus* free and kept in refrigerator from production till consumption.

Key words: Aflatoxins · *Aspergillus flavus* · Experimental contamination yoghurt

INTRODUCTION

Moulds are microscopic organisms that live on plant or animal matter. No one knows how many species of fungi exist, but estimates range from tens of thousands to perhaps 300,000 or more [1].

Mycotoxins are secondary toxic by-products (not essential for survive) of some moulds produced under certain conditions to achieve a competitive advantage for mould over other mould species and bacteria. It is cytotoxic, heat stable toxin for all living cells including higher organisms' cells [2].Among mycotoxins aflatoxins considered as the most harmful toxin to human body, these aflatoxins are produced mainly by *Aspergillus flavus* (*A. flavus*), *A. parasiticus* and some other species of *A. niger* or *A. nomius* [3].

Contamination of human food especially milk and dairy products with *A. flavus* even in trace counts must not pass for consumption, because *A. flavus* even in small counts can multiply and produce aflatoxins if food did not kept in refrigerator.

Therefore this work is adapted for screening of toxicity or toxogenicity of *A. flavus* in food samples as a one of most important species which has public health significance in toxin production.

MATERIALS AND METHODS

Experimental Technique

Fungal Strains: sixteen isolates of *A. flavus* were isolated from cheese and yoghurt samples from previous work [4].

Media: *Aspergillus flavus* Differentiation Medium (ADM) was used to select toxin producing isolates (orange colour at colony biases).Czapak Yeast agar (CYA) was for subculturring of *A. flavus* at three points for 7 days of incubation at 25°C.

Inoculation: Ten ml of distilled water containing 1cm^2 of toxogenic *A. flavus* colony were homogenized and inoculated (after counting of conidia in Thoma chamber and dilution was adjusted to have spores count range of 2:5x10^3). Using sterile syringe, yoghurt samples were inoculated and then examined within the shelf-life. Total *A. flavus* count was done before inoculation of mould and 10 days after incubation [5].

Aflatoxin Detection: Preparations of samples were carried out according to Herzallah [6].

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Extraction: A 20 g subsample was extracted with 40ml acetonitrile and water with ratio (9: 1) using a mechanical shaker for 30 min.

Cleanup: A 2 ml portion of the filtrate was applied to an IMC (ISOLUTE TM Multimode Column, 3 ml capacity, 50 mg mass) that was passed through at a flow rate of 2 ml\min followed with 5 ml acetonitrile with or without water (9: 1) to completely elute the AF. The combined elute was evaporated to dryness and redissolved in 1 ml methanol by sonication and kept in an amber capped vial in a refrigerator at 4 °C until quantitated by HPLC.

Derivatization: An aliquot (300 µl) of sample solution was transferred directly into a HPLC vial and evaporated in a dryer block. n-Hexane (200 µl) and TFA (trifluoroacetic acid) (50 µl) were added to a dried extract, capped and mixed with a vortex mixer for 30 s. The mixture was kept for 5 min, after which 550 µl water± acetonitrile (9:1) was added and again mixed for 30 s. with vortex. The layers were allowed to separate for 10 min. After pipetting out the upper hexane layer, the required volume of aqueous layer was auto injected on to the HPLC column.

Determination of Aflatoxin by HPLC: Aflatoxin was determined using an Agilent 1260 HPLC with a fluorescence detector, operated at an excitation and emission wavelength of 360 and 440 nm, respectively and a 250 x 4.6 mm id Zorbax C18 XDB column with 5 µm particle size. The flow rate was 1.0 mL/min with an oven temperate of 25°C and an injection volume of 20µL. The mobile phase consisted of acetonitrile: methanol: water (13:22:65 v/v). The residues in the real samples were tentatively identified by comparing the retention times (RTs) of the sample peaks with the RTs of the injected standards.

Experimental Conditions

Standard AFL: AFLS standard (B_1, B_2, G_1 and G_2) from Dr. Ehrenstorfer, Augsburg, Germany.

HPLC Conditions:

Column: Zorbax C18 XDB column with 5 µm particle size
Mobile Phase: Acetonitrile: methanol: water (13:22:65 v/v/v)
Flow Rate: 1.0 mL/min
Temperature: Ambient (25 °C)
Detector: UV 360 and 440 nm
Injection volume: 20µL

RESULTS AND DISCUSSION

In referring to our previous work Khalifa *et al.* [4] sixteen isolates of *A. flavus* obtained from examined cheese and yoghurt samples were screened for their ability to produce aflatoxins on food substance. We selected 16 isolates from the 3 types of samples (feta, istanboli cheese and yoghurt) which shown toxin production on Aspergillus Differentiation Medium (ADM) to ensure its accuracy; the inoculated conidial count was adjusted at 2-5 x 10^3/ ml to examined the toxicity of *A. flavus* in yoghurt if it was contaminated by 2 x10^3/ ml and kept out of refrigerator till the end of shelf-life.

According to Table (1) all of the isolates were toxigenic and produced aflatoxins (B_1, B_2, G_1 and G_2) in different concentrations as assessed by HPLC method. Moradi *et al.* [7] detected 333 of 350 *A. flavus* isolates as aflatoxin producers, Dutta and Das [8] found 76% of 198 *A. flavus* strains isolates, were be toxigenic. The yoghurt before inoculation showed no detectable levels of aflatoxins (results not presented).

Isolates of *A. flavus* produced aflatoxin B_1 ranging from 6.59 to 134.78 ppb, AFB_2 was detected in 12 isolates ranging from 2.97 to 5.12 ppb, AFG_1 was positive in 14 isolates with average of 2.37 to 9.53 ppb while, AFG_2 was produced by all strains in level laying between 1.49 to 4.79 ppb.

Comparatively similar findings were detected in dairy and food products by Aycicek *et al.* [9] who detected AFB_1 contamination in samples in a range of <1 to 10 ppb and from <1 to 13 ppb, also, Yousefi *et al.* [10] found *A. flavus* isolets producing aflatoxin B_1 ranging from 0.32 to 12.18 ppb, while 2.3% of isolates produced 18.88 ppb and 0.36 ppb of aflatoxin B_1 and aflatoxin B_2 respectively.

However, higher results were obtained by Zerfridis [11], Hassanin [12] and Aaid [13] in cheese. El-Shanshoury *et al.* [14] detected AFB_1 in contents ranged between 427- 466 µg/kg in cereal grains and peanut and Arrus *et al.* [15] found 4483 ng/g of AFB_1 in Brazilian nuts kept at temperatures of 25-30°C.

it is cleared that not all isolates of *A. flavus* were capable of producing all types of aflatoxins, 10 of 16 isolates produced the 4 aflatoxins [16 and 14] while 2 isolates produced AFB_1 and AFG_2 [17] and AFB_1 not produced by 2 isolates only it is depends on the particular fungal strains and on a particular growth conditions. Wei and Jong [18] found 2 of 9 *A. flavus* strains were capable of producing 4 types of aflatoxins and 9 (100%) could form AFB_1. Moreover, Rodrigues *et al.* [19] able to detect AFBs and AFGs in 8% of *A. flavus* isolates.

Table 1: Screening of aflatoxigenicity of isolated A. flavus strains in artificially contaminated yoghurt samples

	Total *Aspergillus flavus* count/ g		Aflatoxins concentration ppb			
No.	Before inoculation	After inoculation	B_1	B_2	G_1	G_2
1	-	1 x 10^6	-	4.52	2.81	1.49
2	-	1 x 10^7	8.57	3.07	7.22	3.87
3	-	7 x 10^5	10.03	-	2.37	1.99
4	-	1 x 10^6	6.87	-	4.07	2.91
5	-	2 x 10^6	19.65	4.48	2.68	4.79
6	-	2 x 10^7	79.87	-	-	1.77
7	-	2 x 10^7	28.91	-	-	2.21
8	-	2 x 10^6	134.77	2.97	3.03	1.62
9	-	1 x 10^7	9.38	3.07	3.83	2.04
10	-	3.5 x 10^6	27.06	5.12	9.53	1.80
11	-	3 x 10^6	9.91	3.81	2.68	1.77
12	-	1 x 10^6	12.46	3.19	4.34	2.72
13	-	2 x 10^5	-	4.84	4.38	1.81
14	-	1 x 10^5	8.31	3.43	3.29	2.77
15	-	1.5 x 10^5	6.59	3.88	2.93	4.18
16	-	1 x 10^5	11.77	4.99	2.84	2.05

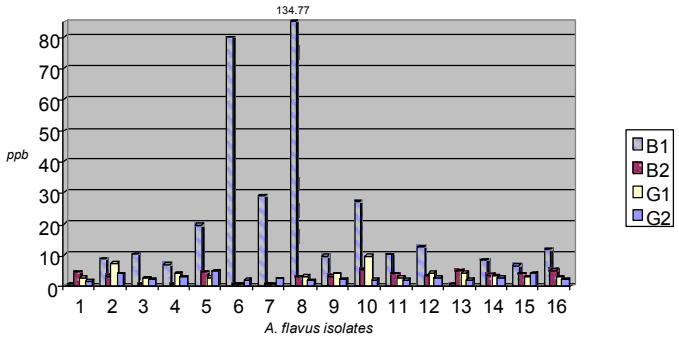

Fig. 1: Screening of aflatoxigenicity of isolated *A. flavus* strains

CONCLUSION

Increasing temperature during storage of yoghurt resulted in an increase in aflatoxin production ability of *A. flavus* especially with high relative humidity. Therefore yoghurt must be kept in refrigeration temperature till consumption.

REFERENCES

1. United States Department of Agriculture (USDA), 2007. NationalAgriculturalStatistics Service. Census of Agriculture. B-36 APPENDIX B.
2. Pitt, J.I. and A.D. Hocking, 1997. Fungi and Food Spoilage Book 3rd Ed., Academic Press, New York, London, Toronto, Montreal, Tokyo.
3. Diaz, S., L. Dominguez, J. Prieta, J. Blanco and M.A. Moreno, 1995. Application of a diphasic dialysis membrane procedure for surveying occurrence ofaflatoxin M1 incommercialmilk. J. Agric. Food Chem., 43: 2678-80.
4. Khalifa, Marwa, Maha, Al-Ashmawy, A. Abdel-Khalik and M. El-Sherbini, 2013. Mycological Evaluation of Serving some Dairy Products with Special Reference to Mycotoxins Production in Azhar University Student Hostels. World Journal of Dairy and Food Sinces, 8: 165-170.
5. Garcia, M.E., J.L. Blanco and G. Suarez, 1995. Influence of inoculum size on aflatoxin production in home-made yoghurt. Mycotoxin Research, 11: 62-68.
6. Herzallah, S., 2009. Determination of aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors. Food Chemistry, 114: 1141-1146.
7. Moradi, M., R. Dargahi, A. Sherafati, S.R. Fani, E. Sedaghati, M. Mohammadi-Moghaddam and M. Mirabolfathy, 2012. Screening of Aspergillus flavus isolates in Pistachio producing areas of Iran. Acta Hort. (ISHS) 963: 91-93.http://www.actahort.org/books/963/963_14.htm.

8. Dutta, T.K. and P. Das, 2001. Isolation of aflatoxigenic strains of Aspergillus and detection of aflatoxin B1 from feeds in India. Mycopathologia, 151: 29-23.
9. Aycicek, H., A. Aksoy and S. Saygi, 2005. Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. Food Control, 16: 263-266.
10. Yousefi, S., S. Dadgar, M. Safara and F. Zaini, 2009. Aflatoxin production by Aspergillus flavus isolates from green-tiger shrimps (Penaeussemisulcatus). Iranian Journal of Microbiology, 1: 18-22.
11. Zerfiridis, G.K., 1985. Potential aflatoxin hazards to human health from direct mold growth on Teleme cheese. J. dairy Sci., 68: 2184-2188.
12. Hassanin, Nagwa, 1994. Stability of aflatoxin M1 duringmanu facture and storage of yoghurt, yoghurt-cheese and acidified milk. J. Sci. Food Agric., 65: 31-34.
13. Aiad, A.A., 2002. Criteria for evaluation of locally manufactured dairy products. Ph.D. thesis, Fac. Vet. Med., Alex. Univ., Egypt.
14. Abd El-Raheem, R. El-Shanshoury, Sabha M. El-Sabbagh, H. Emaraand Hebaallah E. Saba1, 2014. Occurrence of moulds, toxicogenic capability of Aspergillus flavus and levels of aflatoxins in maize, wheat, rice and peanut from markets in central delta provinces, Egypt. Int. J. Curr. Microbiol. App. Sci., 3: 852-865.
15. Arrusa, K., G. Blanka, D. Abramsonb, R. Clearc and R.A. Holley, 2015. Aflatoxin production by Aspergillus flavus in Brazil nuts. Journal of Stored Products Research, 41: 513-527.
16. Abou-Zeid, A., M. Metwally and B. Farid, 1997. Physiological and hepatotoxic studies on fungal aflatoxin isolated from Egyptian cereals. Egyptian Journal of Microbiology, 32: 83-98.
17. Davis, N.D., U.L. Diener and D.W. Eldridge, 1966. Production of Aflatoxins B1 and G1 by Aspergillus flavus in a Semisynthetic Medium. Applied Microbiology, 14(3): 378-380.
18. Wei, D.L. and S.C. Jong, 1986. Production of aflatoxins by strains of the Aspergillus flavus group maintained in ATCC. J. Mycopathologia, 93: 19-24.
19. Rodrigues, Paula, A. Venâncio, Zofia, Kozakiewicz and N. Lima, 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus Section Flavi isolated from Portuguese almonds. International Journal of Food Microbiology, 129: 187-193.