Journal of Animal and Feed Sciences, 20, 2011, 300–309

Detoxification of aflatoxin B_1 and change in microflora pattern by probiotic *in vitro* fermentation of broiler feed*

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(Received 10 February 2011; revised version 24 May 2011; accepted 18 June 2011)

ABSTRACT

The aim of this study was to determine the influence of spontaneous fermentation and that with the use of probiotic bacteria and yeast on aflatoxin B_1 concentration and the microflora pattern during fermentation. The probiotic preparation used contained bacteria resistant to gastric juice and bile: *Lactobacillus paracasei* LOCK 0920*, Lactobacillus brevis* LOCK 0944*, Lactobacillus plantarum* LOCK 0945*,* as well as live yeasts *Saccharomyces cerevisiae* LOCK 0140 of high fermenting capacity. After 6-h fermentation with the probiotic, in feed mixture with a low concentration of aflatoxin B_1 (1 mg/kg), the amount of aflatoxin B_1 decreased by 55%. In the case of a high concentration (5 mg/kg) the decrease in aflatoxin B_1 was about 39%. This tendency was sustained during the following hours of incubation (12^{th} and 24^{th} h). The application of probiotic bacteria and yeasts resulted in the reduction of aerobic spore forming bacteria.

KEY WORDS: broiler feed, aflatoxin B_1, incubation, probiotic preparation, detoxification

* Supported by the State Committee for Scientific Research, Grant No. R12 028 01
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INTRODUCTION

The data published by FAO in 2001 showed that 25% of agricultural products were contaminated with mycotoxins. Aflatoxins, particularly aflatoxin B_1 (AFB), are considered to be the most important of the mycotoxins due to their high toxicity and they are still of major concern to the feed industry and farmers as many raw materials which are used as components of animal feeds are prone to contamination. Poultry species differ in their susceptibility to aflatoxin B_1, young birds are more sensitive than older ones (FAO/WHO, 2001; Fraga et al., 2007).

The adverse effects of mycotoxins involves their mutagenic, carcinogenic (especially to kidneys and liver), teratogenic and oestrogen immunosuppressive effects. Aflatoxin B_1 is one of the strongest carcinogens and it was included by WHO and the International Agency for Research on Cancer in the list of carcinogenic substances Group I, i.e. substances with confirmed carcinogenic effect in humans (JECFA, 1998; FAO/WHO/UNEP, 1999).

Plant materials already contaminated with mycotoxins should be detoxified. It is a common practice to add different adsorbents to animal feeds to prevent the negative effects of mycotoxins (Huwig et al., 2001). Also a microbial degradation is one of the well-known strategies for the management of mycotoxins in foods and feeds. Among the different potentially decontaminating microorganisms, *Saccharomyces cerevisiae* and lactic acid bacteria represent unique species which are widely used in food fermentation and preservation (Baptista et al., 2004; He et al., 2010); these species are also components of probiotic preparations used in broiler production (Line et al., 1998; Patterson and Burkholder, 2003; Smulikowska et al., 2005).

The aim of this study was to determine the efficiency of a probiotic preparation composed of defined strains of lactic acid bacteria, yeasts and a yucca extract in degradation of aflatoxin B_1 in broiler feed mixture during incubation *in vitro*.

MATERIAL AND METHODS

Material

Wheat grain was inoculated with the strain of *Aspergillus flavus* K30 (Collection of Pure Cultures, Institute of Food Technology of Plant Origin, Poznań University of Life Sciences) and incubated to obtain high concentration of aflatoxin B_1 in accordance with a procedure described by Xiao et al. (1996). Twenty Erlenmeyer

flasks (500 ml), each containing 30 g of feed-grade wheat and 30 ml of distilled water, were autoclaved for 30 min at 120ºC and then inoculated with *Aspergillus flavus* K30. The culture was maintained at 30ºC in a dark room. The fermentation was terminated on day 12 of incubation, the contaminated wheat was dried for 72 h, ground and the aflatoxin B_1 (AFB) concentration was determined. The diet was formulated to meet or exceed the requirements of broilers (Table 1), adequate amount of contaminated wheat was substituted for uncontaminated one to obtain the final concentration of 1 or 5 mg of aflatoxin B_1 per kg of diet

Table 1. Composition of the feed mixture for broiler chickens, g/kg $^{-1}$

Component	g/kg
Wheat	330.7
Soyabean meal	380.6
Maize	200
Limestone	8.5
Dicalcium phosphate	18
NaCl	3
Rapeseed oil	50
Vitamin-mineral premix	5
Wheat starch or probiotic	1
L-lysine (78%)	1
DL-methionine (98%)	1.2
Feed enzyme	1

[1] concentration of aflatoxin B_1 in feed mixture was 1 mg/kg or 5 mg/kg (see Material and methods)

The probiotic preparation (PP) used contained (per 1 kg): 10^{10} of *Lactobacillus* cells (*L. paracasei* LOCK 0920, *L. brevis* LOCK 0944 and *L. plantarum* LOCK 0945), 10^6 of yeast *Saccharomyces cerevisiae* LOCK 0140 cells and 50 g of *Yucca schidigera* extract. The strains derived from Centre of Industrial Microorganisms (LOCK), Institute of Fermentation Technology and Microbiology, Technical University of Lodz in Poland. The strains were formerly used in broiler trials (Smulikowska et al., 2005), the preparation possess full probiotic documentation and is licensed (Michałowski et al., 2004).

Fermentation

The 1 kg samples of broiler diets with different aflatoxin B_1 concentration were closed tightly in polyethylene bags and sterilized by radiation with doses ranging 25 kGy at ambient temperature. Radiation was carried out by ^{60}Co γ-rays at a dose rate of 0.2 Gy s^{-1}, Fricke dosimetry being employed. Sterilized control samples were kept tightly closed at 37^{0}C and sampled after 6, 12 and 24 h for aflatoxin B_1 analysis.

The one kg samples of each unsterilized diet without supplement (spontaneous fermentation) or with addition of 1 g PP per kg (probiotic fermentation) was mixed with distilled water in a proportion of 1:1.5 (w/w) and incubated at 37ºC in an aerobic atmosphere for 24 h. Fermentation was made in triplicate. After 6, 12 and 24 h of fermentation the 10 g of each diet were sampled for microbiological and aflatoxin B_1 analysis.

Analytical procedures

The concentration of aflatoxin B_1 in wheat grain and in diets was measured with an enzyme linked immunosorbent assay (ELISA direct competitive immunoassay with horseradish peroxidase conjugate) using a commercial ELISA kits (AgraQuant Aflatoxin, Romer Labs Diagnostic, Singapore) according to the procedure described in the Agra-Quant Assay kit manual. In brief: aflatoxin B_1 was extracted from 10 g of sample with 50 ml methanol:water (70:30 v/v). Independently, 100 µl of conjugate mixture were added to each well containing standards and previously filtered samples (50 µl). After mixing, 50 µl were transferred to wells containing antibodies and incubated for 15 min. Then, the content was eliminated, and the wells were washed five times with deionized water. Any excess of water was discarded and the wells were dried. Substrate (50 µl) was then added to each well, incubated for 5 min, and the reaction was stopped by adding 50 µl stop solution. Optic density was read with an UVM 340 microplate reader (Asys, Austria) using a 450 nm filter. The aflatoxin B_1 concentration was calculated by extrapolating the optic density with the respective calibration curve (Zheng et al., 2005).

Microbiological analyses

The microbiological analyses were carried out according to the Polish Standard of PN-ISO (PN-EN ISO 4833, 2004). The following bacteria species were identified: *Lactobacillus* on MRS agar medium (Merck), using a double-layer technique and anaerobic incubation at 35ºC/72 h; *Clostridium* on TSC agar (Merck) and anaerobic incubation at 37ºC/18-24 h; *Pseudomonas* on PM5 agar (BTL) and anaerobic incubation at 37ºC/24 h; the *Coli* group on VRBL agar (Merck) and aerobic incubation at 35ºC/24 h; total number of anaerobic bacteria on Plate Count agar (Merck) and anaerobic incubation at 35ºC/24 h, and total number of yeasts on YGC agar (Merck) and aerobic incubation at 20ºC/120 h. Anaerobic bacteria were cultured in the anaerobic atmosphere of H:N:CO_2 1:8:1 (Anaerobic Workstation Concept 400, Biotrace Int.). The specific morphology of cells was checked under a microscope (Olympus CX-41). Each determination was done in triplicate. The results are presented as colony forming units (cfu) per gram samples.

Calculations and statistical analysis

Data were collected in triplicate and subjected to three-way analysis of variance by the general linear model (GLM) procedure of Statgraphics Centurion XVI. Statistical significance was accepted at $P<0.05$. In case when significant difference was found, post-hoc Tuckey HSD difference test was used to compare between mean differences. For bacterial counts the analysis of variance was carried out on data submitted to logarithmic transformation.

RESULTS

After 6 h keeping at 37°C in diets that had been previously subject to radiation sterilization the amount of AFB was reduced to 0.89 mg/kg (by 10%) in the sample containing initially 1 mg AFB /kg , and to 4.57 mg/kg (by 8.5%) in the sample with the higher dose of the toxin. The concentration of AFB in irradiated feed did not change during further 24 h (Figure 1). The concentration of AFB decreased after 6 h of incubation at 37°C in non supplemented diet by 16-20% and did not decrease substantially during further 12 and 24 h of incubation (Figure 1, Table 2).

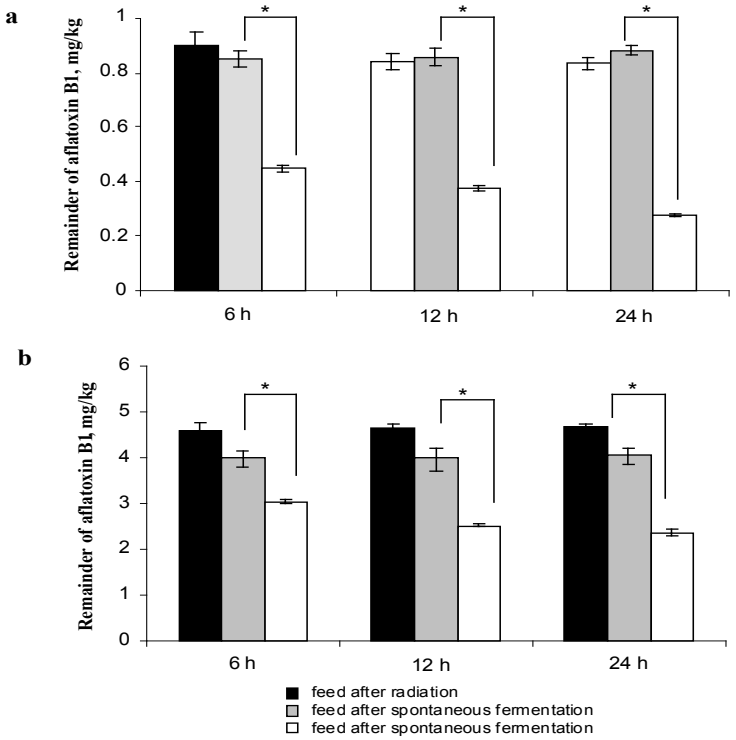

Figure 1. Concentration of aflatoxin B_1 in the diet (values are means of 3 replicates ± SEM): a - initial concentration 1 mg/kg ; b - initial concentration 5 mg/kg. Asterisks indicate significant differences ($P<0.05$)

Table 2. Main effects of supplementation with probiotic preparation, dietary aflatoxin B_1 level and time of incubation on bacterial and yeasts counts, log CFU/g diet and on aflatoxin B_1 concentration in feed, mg/kg

Main effect	Total bacteria	Anaerobic spore forming bacteria	*Lactobacillus*	Total yeasts	Aflatoxin B_1
Aflatoxin B_1 level (A)					
1 mg/kg	7.62	3.02	6.44	4.92	0.77^{B}
5 mg/kg	7.55	3.32	6.27	4.82	4.07^{A}
SEM	0.341	0.418	0.246	0.210	0.018
Probiotic (P) supplement					
without probiotic	7.12	4.10^{a}	4.33^{A}	2.87^{A}	2.58^{A}
with probiotic	8.05	2.24^{b}	8.37^{B}	6.87^{B}	1.87^{B}
SEM	0.341	0.418	0.246	0.210	0.022
Time of incubation (T)					
0	6.62^{A}	3.26	4.93^{A}	3.72^{A}	3.00^{a}
6 h	7.35^{B}	3.08	6.08^{B}	4.52^{B}	2.29^{b}
12 h	7.95^{C}	3.13	6.98^{C}	5.18^{C}	2.20^{b}
24 h	8.42^{D}	3.22	7.42^{D}	6.07^{D}	2.17^{b}
SEM	0.095	0.079	0.135	0.100	0.087
Interactions					
A x P	Ns	Ns	Ns	Ns	0.001
A x T	Ns	0.05	Ns	Ns	0.001
P x T	>0.001	>0.001	>0.001	>0.001	0.001

$^{a,b, A,B}$ within columns, for each main effect, means with different superscript letters are significantly different at: ^{a,b} $P < 0.05$; ^{A,B} $P < 0.01$

However, in broiler diets with initial concentration of 1 mg AFB/kg supplemented with probiotic after 6 h of incubation at 37°C the concentration of AFB was reduced to 0.45 mg/kg (by 55%) and in diet with 5 mg AFB/kg to and 3.03 mg/kg (by 39%), respectively. Aflatoxin B_1 concentration was reduced further during prolonged incubation, and after 24 h incubation it decreased by 73% in the sample containing initially 1 mg AFB /kg and by 53% in the sample with a higher dose of AFB (the interaction AxT and PxT significant; Table 2). The differences were statistically significant ($P<0.05$) in comparison with the irradiated sample and sample without probiotic (Figure 1).

Main effects of experimental treatments on bacteria and yeasts number was shown in Table 2. Initial dietary aflatoxin B_1 did not affect bacteria and yeasts counts. *Lactobacillus*, total bacteria and yeasts counts increased ($P<0.01$) during incubation in diets without and with probiotic preparation. However, the increase in bacterial counts was higher by 2 orders of magnitude, in yeasts counts by 4 orders of magnitude in the diets supplemented with the probiotic preparation (interactions probiotic x time were highly significant).

The inhibition of viable aerobic spore forming bacteria increased in unsupplemented diets and decreased in the diets supplemented with probiotic

(interactions probiotic x time were highly significant). Significant interaction ($P<0.05$) was found also between initial dietary aflatoxin B_1 level and time of incubation for anaerobic spore forming bacteria, their numbers decreased in the diet containing 1 mg aflatoxin B_1 after 6 h, than slightly increased, while in the diet containing 5 mg aflatoxin B_1 it increased with time of incubation (Table 2).

In both diets initially the bacteria from the *Coli* group as well as *Pseudomonas* and *Clostridium* were present at average amounts 2.0, 4.1 and 1.0 log CFU/g, respectively, but they were not found after 6 or 12 h of incubation. The effect was connected neither with aflatoxin B_1 concentration nor with the presence of probiotic.

DISCUSSION

Broiler feed used in the current study was heavily contaminated with aflatoxin B_1, much above the level 0.02 mg/kg permitted for poultry (Directive 2002/32/EC). Probiotic preparations are aimed to populate the intestinal tract with bacteria (Patterson and Burkholder, 2003) or yeasts (Line et al., 1998), which influence the environment of the gut and favour the establishment of beneficial rather than detrimental species. The current study shows that during *in vitro* fermentation, despite the high or very high aflatoxin B_1 (AFB) level in broiler feed, the lactic acid bacteria (LAB) and yeasts multiplyied considerably in feed with addition of probiotic in comparison to feed without the probiotic. The influence of applied probiotic on the *Clostridium* growth was inconclusive, but in our earlier study the probiotic added to the feed reduced significantly *Clostridium* number in excreta of 3-week-old chickens (Smulikowska et al., 2005).

In the present study the concentration of aflatoxin B_1 in a feed mixture decreased considerably due to probiotic activity. From practical point of view the most important is the time of 6 h of fermentation, as the passage rate of feed in broilers ranges between 4-8 h. During first 6 h of fermentation in the feed with a low AFB concentration the amount of aflatoxin B_1 decreased by 55%, in the feed with the high AFB concentration (5 mg/kg) the reduction in aflatoxin B_1 equalled about 39%. The prolonged time of incubation (12 and 24 h) resulted in further decrease of AFB concentration. After 24 h of fermentation the amount of aflatoxin B_1 was reduced by 73% in samples with lower and by 53% in samples with a higher aflatoxin B_1 concentration.

Biological detoxification of mycotoxins in food, raw products, mixed protein feeds and also in human and animal organisms is a novel and very promising process. Among the organisms that have been used for mycotoxin elimination, are: *Acinetobacter calcoaceticus* bacteria (Kusumaningtyas et al., 2006), *Aspergillus*

(Wiseman and Marth, 1981), *Alternaria, Botrytis, Cladosporium, Phaffia, Penicillum* and *Rhizopus* moulds (Niderkorn et al., 2006), lactic acid bacteria of the *Lactobacillus* strains (El-Nezami et al., 1998) and *Saccharomyces* yeasts (Kusumaningtyas et al., 2006).

Shetty and Jespersen (2006) examining the ability to bind aflatoxin B_1 by different strains of lactic acid bacteria and yeast obtained the best results with strains of *Saccharomyces cerevisiae*, *Candida krusei, Lactobacillus plantarum* and *Lactobacillus fermentum*, which eliminated 60% or more of the aflatoxin B_1 from saline. Peltonen et al. (2001) shown that a fairly high efficiency (50%) of binding of aflatoxin B_1 from a buffered saline solution is displayed by strains of *Lactobacillus rhamnosus* and *Lactobacillus amylovorus*, while *Lactococcus lactis* spp. *cremoris*, *Bifidobacterium animalis* and *Bifidobacterium lactis* were slightly less efficient (40-50%). Experiments on decontaminating chicken feed containing aflatoxin B_1 by using *Saccharomyces cerevisiae*, *Rhizopus oligosporus* or both species were conducted by Kusumaningtyas et al. (2006). Both separate and combined use of these organisms yields similar results in reducing the concentration of aflatoxin B_1 being most evident (over 60% reduction) after 5 days of action.

Nevertheless, special interest has been paid to lactic acid bacteria due to their favourable influence on animal and human organisms and the widespread use in the production of fermented food and probiotic preparations. These bacteria inhibit both the growth of moulds and their production of mycotoxins (Shetty and Jespersen, 2006). Probiotics also can improve the feed utilization by means of specific hydrolytic enzymes production that decompose carbohydrates as well as can increase the activity of the host's enzymes, such as β-galactosidases, saccharase and maltase (Ŝtyriak et al., 2001).

In order to investigate the mechanisms which account for the removal of mycotoxins by LAB, the effects of viable and heat inactivated bacteria were compared in a number of studies (El-Nezami et al., 1998; Fink-Gremmels, 1999). Additionally, the bacteria were treated with enzymes (such as pronase E and lipase) or periodate, which cause alterations to the structure of the cell walls (Haskard et al., 2001; Lahtinen et al., 2001). On the basis of the results obtained in these experiments, it was postulated that the removal of aflatoxin B_1 and zearalenone is due to the non-covalent binding of the toxins to the carbohydrate moieties of the cell walls. However, since a decrease in their toxic effects was also observed in the case of cytosolic preparations of LAB, it was hypothesized that other mechanisms (e.g., interactions with short chain fatty acids) might also play a role (Allameh et al., 2005).

Contamination of broiler feeds with AFB can occur occasionally irrespective of the grain quality monitoring procedures (Fraga et al., 2007). The results of

the present study indicate that the probiotic preparation containing LAB bacteria, *Saccharomyces cerevisiae* yeasts and *Yucca shidigera* extract aimed at beneficial modulation of gut microbiota, can also effectively decrease the level of AFB during 6 h fermentation of feed mixture. The *in vivo* experiment on broilers is needed to confirm a usefulness of the preparation in protection of broilers against the occasional AFB contamination.

ACKNOWLEDGEMENTS

The skilful assistance of dr M. Taciak in statistical analysis of results is highly appreciated.

REFERENCES

Allameh A., Safamehr A., Mirhandi S., Shivazad M., Razzaghi-Abyaneh M., Afshar-Naderi A., 2005. Evaluation of biochemical and production parameters of broiler chicks fed ammonia treated aflatoxin contaminated maize grains. Anim. Feed Sci. Tech. 122, 289-301

Baptista A.S., Horii J., Calori-Domingeus M.A., da Gloria E.M., Salgado J.M., Vizioli M.R., 2004. The capacity of mannooligosaccharides thermolysed yeast and active yeast to attenuate aflatoxicosis. World J. Microbiol. Biotechnol. 20, 475-481

Directive 2002/32/EC of European Parliament and of the Council of 7 May 2002 on Undesirable Substances in Animal Feed

El-Nezami H., Kankaanpaa P., Salminen S., Ahokas J., 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B_1. Food Chem. Toxicol. 36, 321-326

FAO/WHO, 2001. Safety evaluation of certain mycotoxins in food. Joint FAO/WHO Expert Committee on Food Additives, 56th Meeting Geneva

FAO/WHO/UNEP, 1999. Mycotoxin prevention and decontamination, HACCP and its mycotoxin control potential: An evaluation of ochratoxin A in coffee production. Third Joint FAO/WHO/UNEP International Conference On Mycotoxins, 6 c: 1–13. Available at: ftp://ftp.fao.org/es/esn/food/ myco6c.pdf

Fink-Gremmels J., 1999. Mycotoxins: their impact on human and animal health. Vet. Q. 21, 115-120

Fraga M., Curvella F., Gatti M., Cavaglieri L., Dalcero A., 2007. Potential aflatoxin and ochratoxin A production by *Aspergillus* species in poultry feed processing. Vet. Res. Commun. 31, 343-353

Haskard C.A., El-Nezami H., Kankaanpaa P., Salminen S., Ahokas J., 2001. Surface binding of aflatoxin B_1 by lactic acid bacteria. Appl. Environ. Microbiol. 67, 3086-3091

He J., Zhou T., Young J.C., Boland G.J., Scott P.M., 2010. Chemical and biological transformations for detoxification of mycotoxins in human and animal food chains: a review. Trends Food Sci. Technol. 21, 67-76

Huwig A., Freimund S., Käppeli O., Dutler H., 2001. Mycotoxin detoxication of animal feed by different adsorbents. Toxicol. Lett. 122, 179-188

JECFA, 1998. Safety evaluation of certain food additives and contaminants. Aflatoxins. WHO Food Additives Series 40, pp. 897-913

Kusumaningtyas E., Widiastuti R., Maryam R., 2006. Reduction of aflatoxin B_1 in chicken feed using *Saccharomyces cerevisiae*, *Rhizopus oligosporus* and their combination. Mycopathologia 162, 307-311

Lahtinen S.J., Haskard C.A., Ouwehand A.C., Salminen S., Ahokas J., 2001. Binding of aflatoxin B1 to cell wall components of *Lactobacillus rhamnosus* strain GG. Food Addit. Contam. 2, 158-164

Line E.J., Bailey J.S., Cox N.A., Stern N.J., Tompkins T., 1998. Effect of yeast-supplemented feed on Salmonella and Campylobacter populations in broilers. Poultry Sci. 77, 405-410

Michałowski P., Libudzisz Z., Śliżewska K., Klewicka E., Motyl I., Zabielski R., 2004. Probiotic Growth Promoter for Poultry. Licence number P-371363

Niderkorn V., Boudra H., Morgavi D.P., 2006. Binding of Fusarium mycotoxins by fermentative bacteria in vitro. J. Appl. Mirobiol. 101, 849-856

Patterson J.A., Burkholder K.M., 2003. Application of prebiotics and probiotics in poultry production. Poultry Sci. 82, 627-631

Peltonen K., El-Nezami H., Haskard C., Ahokas J., Salminen S., 2001. Aflatoxin B1 binding by dairy strains of lactic acid bacteria and bifidobacteria. J. Dairy Sci. 84, 2152-2156

PN-EN ISO 4833, 2004. Microbiology of Food and Animal Feedingstuffs. Horizontal Method for the Enumeration of Microorganisms

Shetty P.H., Jespersen L., 2006. Saccharomyces cerevisiae and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends Food Sci. Technol. 17, 48-55

Smulikowska S., Śliżewska K., Biernasiak J., Mieczkowska A., Michałowski P., 2005. The effect of a probiotic composed of Lactobacillus and yeasts, and of flavomycin on the performance and faecal microflora of broiler chickens. J. Anim Feed Sci. 14, Suppl. 1, 483-485

Statgraphics Centurion ver. XVI 1.03, 1982-2010. StatPoint Technologies.Inc

Ŝtyriak I., Čonková E., Kmet V., Böhm J., Razzazi E., 2001. The use of yeast for microbial degradation of some selected mycotoxin. Mycotoxin Res. 174 (2), 24-27

Wiseman D.W., Marth E.H., 1981. Growth and aflatoxin production by Aspergillus parasiticus when in presence of Streptococcus lactis. Mycopathologia 63, 49-56

Xiao H., Marquardt R.R., Abramson D., Frohlich A.A., 1996. Metabolites of mycotoxin in rat urine and in a culture of Aspergillus ochraceus. Appl. Environ. Microbiol. 62, 648-655

Zheng Z., Humphrey C.W., King R.S., Richard J.L., 2005. Validation of an ELISA test kit for the detection of total aflatoxins in grain and grain products by comparison with HPLC. Mycopathologia 159, 255-263