J.Animal and Poultry Prod., Mansoura Univ., Vol.8(5): 91 - 95 ,2017

Inhibition of Aflatoxin B_1 Production by Bacteria

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ABSTRACT

Aflatoxins (AFs) contamination in food is a serious problem in the world. Aflatoxin B_1 (AFB_1), produced by *Aspergillusflavus*,is secondary metabolite, highly toxic and carcinogenic. The reduction of AFs contamination in food and feed products could be achieved by some microorganisms. In this study,*Lactobacillus plantarum* ATCC 14917, *Lactobacillus curvatus* ATCC 1136, *Bacillus megateirum* A.F.10, *Bacillus subtitles* A.F.12and commercial probiotic were used to inhibit AFB_1 production on yeast extract sucrose medium (YES) and on corn. All bacterial strain inhibited*A. flavus*production of AFB_1 on YES media and corn. Also, commercial probiotics hadthe ability to inhibit *A. flavus* growth and its production of AFB_1 on corn. The most effective bacteria was*B. megaterium* A.F.10. It inhibited the fungal growth with 12 mm inhibition zone and inhibits AFB_1 production 100% by HPLC on YES media. Determination of AFB_1 production by HPLC on corn showed that *B. megaterium* inhibitedthe production by 69.81% followed by commercial probiotics (59.62%).Commercial probiotics and *B. megaterium* had a synergistic effect in inhibition ofAFB_1 production and *in vitro* digestion of corn.

Keywords: *Aspergillusflavus*, aflatoxin, *Lactobacilli,*inhibition.

INTRODUCTION

Aflatoxins (AFs) are mycotoxins produced as secondary metabolites by *Aspergillusflavus* and *Aspergillusparasiticus*. These fungi grow on certain foods and feeds producingAFs.AFB_1 is one of the most serious mycotoxins for human and livestock. The AFs contaminated diets lead to many hazard effects on humans and animals (death; reduce the production and reproduction; mutagenic, carcinogenic and teratogenic effects and immunotoxicity). Several strategies have been proposed to inactivate and detoxify AFs including physical, biological and chemical methods (FAO, 2001; Shehata, 2002; Zaki *et al*., 2008; Shehata 2010, 2012; Shehata *et al*., 2009; Eckhardt *et al*., 2014 and El-Melegy *et al*., 2015).

Chemical and physical detoxification methods have undesirable health effects and high cost of equipment (Basappa and Shantha, 1996). Therefore, the biological methods by using beneficial bacteria are suitable and safe to reduce AFs in contaminated media (Phillips *et al*., 1994 and Farzaneh *et al*., 2012). Probiotic bacteria species belonging to *Lactobacillus*, *Streptococcus* and*Enterococcus* have been reported to enhance the beneficial intestinal probiotic microflora, animal performance and health (Fritts *et al*., 2000 and Transito *et al*., 2011).

Moreover, the probiotic protect against food mutagens such as heterocyclic amines, nitroso-compounds and AFs. Probiotics inhibit the pathogenic bacteria in gastrointestinal tract of animals and humans. The application of bacteria for the AFs remediation take short time.Microorganisms (yeasts, molds and bacteria) have been screened for their ability to modify or inactivate mycotoxins.Inhibition of mold growth in the presence of lactic acid bacteria (LAB) has been reported. Effect of several LABon mold growth and mycotoxin production has also been described (Zaki *et al*., 1992 and Gomah *et al*., 2009).

AFs accumulation in potato dextrose broth medium and liquid minimal medium was almost totally (more than 98 %) inhibited by co-cultivation with *Bacillusmegaterium*. Growth was also reduced (Qing Kong*et al*., 2014).*Latobacilluscurvatus*HBO2 could inhibit growth of fungi. It was cultured on doubled-layered agar or in liquid anerobic cultivation medium (Dong-mei*et al*., 2008). Also,*Bacillus subtilis* could considerably remediate aflatoxin B_1 from nutrient broth culture and pistachio nut by 85.66% and 95% respectively (Farzaneh*et al*., (2012).

The aim of the current study was to: i-inhibit*A. flavus* growth and mycotoxin productionon YES medium& corn, ii- improvement*in vitro* dry and organic matter disappearance of corn by different bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions:

(a) Commercial probioticwas supporting of general Pharma Company. It is considered as a probiotic contain the following ingredients: *Lactobacillus sp.* and *Bacillus subtilus*(4 x 10^{12} CFU/g), (b) *Lactobacillus plantarum* ATCC 14917, (c)*Lactobacillus curvatus* ATCC 1136, (d) *Bacillus subtiles* A.F 12 and (e) *Bacillus megaterium* A.F 10 obtained from Dr. Abdel-Salam, A.F., Regional Center for Food and Feed, ARC, Giza, Egypt (Table 1). The purity of the strains was confirmed by Gram-staining. The *L. plantarum* and *L. curvatus* were grown in DeMan Rogosa Sharpe (MRS) agar at 37°C for 24h.;they were stored in MRS broth at -20°C containing 20% (v/v) glycerol. *B. subtiles* and *B. megaterium* were grown in nutrient agar and stored at -20°C in nutrient broth containing 20% glycerol.

Standard bacterial inoculants:

Standard bacterial inoculants were prepared by inoculation 1% v/v of *L.plantarum, L. curvatus*, *B.megaterium* and *Bacillus subtiles* in conical flask containing 50 mL of MRS broth pH 6.2 for 24 h. at 37ºC. Achieved viable cells count (CFU) was determined by serial dilution and subsequent enumeration on MRS agar.

Inhibition of *A. flavus* growth on solid media by bacterial strains:

The bacteria were grown on nutrient agar medium for 24 h. at 37ºC. Agar discs 7 mm in diameter were cut off by a cork borer and transferred to the

surface of agar plates freshly cultivated by *A. flavus* on CzapexDox agar media. The inhibition zone diameter was determined according to Valgas *et al*., (2007).

Table 1.Sources of tested bacteria.

Tested bacteria	Source	Accession number
1- Commercial probiotic (*Lactobacillus sp.* and *Bacillus subtilus)*	Pharma company	-
2- *Lactobacillus plantarum*	Mercens*	ATCC 14917
3- *Lactobacillus curvatus*	Mercens	ATCC 1136
4- Bacillus megaterium	Mercens	A.F. 10
5- Bacillus subtitles	Mercens	A.F. 12

*** Obtainedfrom Dr. Abdel-Salam, A.F., Regional Center for Food and Feed, ARC, Giza, Egypt**

Inhibition of aflatoxin B_1 production by *A. flavus* on YES medium:

The ability of commercial probiotic,*L. plantarum,L. curvatus*, *Bacillus subtiles* and *B. megaterium*to inhibit aflatoxin B_1 production by *A. flavus*strain NRRL 3145 was obtained from central lab. Of residues in agriculture products, Agric. Pesticides research center, Dokki, Egypt, and investigated by the stimultaneous antagonism assay as described by Munimbazi and Bullerman (1998). Hundred ml portions of YES medium were sterilized at 121ºC for 15 minute in 250 mLErlennmeyer flasks. Six treatments : 1-*A. flavus* + 10 g commercial probiotics (4×10^{12}cells/g); 2-*A. flavus* + 10 mL *L. plantarum* (8×10^{9}cfu/mL); 3- *A. flavus* + 10 mL *L. curvatus*(8×10^{9}cfu/mL; 4-*A. flavus* + 10 mL *Bacillus megaterium*(8×10^{9}cfu/mL); 5- *A. flavus* + 10 mL*Bacillus subtilis*(8×10^{9}cfu/mL) and 6-*A. flavus* only (control) were used, three flasks /each treatment. *A. flavus*of each treatment was 1 mL of fungal spores suspension containing 10^{7}spores/mL.All flasks were incubated at 28º C and analyzed for aflatoxin B_1 production after 8 days of incubation.

Inhibition of aflatoxin B_1 production by *A. flavus* on corn:

Hundred g of free aflatoxincorn was added to 400 mL tap water in Erlenmeyer flasks (2 L.)(3 replicate/each treatment). The content was heated to start boiling and the free water was drained. All flasks were autoclaved at 121ºC for 15 minute. The flasks were left until cooling, and then inoculated with 20 mL of *A. flavus* spores suspension containing 10^{7} spores/mL.The treatments were: 1- *A. flavus* + 10 g commercial probiotics;2- *A. flavus* + 10 mL*L. plantarum;* 3-*A. flavus + 10 mL L. curvatis;* 4- *A. flavus + 10 mL B. megaterium*; 5-*A. flavus* + 10 mL *Bacillus subtillus* and 6- *A. flavus*only (control). All flasks were incubated at 28º C and analyzed for aflatoxin B_1 production after 8 days of incubation.

The synergistic effect of mixed probiotics + *B. megaterium* on inhibition of AFB_1 production wascarried out. The treatments were: 1- *A. flavus* + 10 g commercial probiotics; 2- *A. flavus* + 10 mL*B. megaterium*; 3- *A. flavus* + 10 g commercial probiotics + 10 mL*B. megaterium* and 4- *A. flavus* only (control). All flasks were incubated at 28º C and analyzed for aflatoxin B_1 production after 8 days of incubation.

Determination of AFB_1 on YESmedia and corn:

At the end of incubation period (8 days), AFB_1 in YES medium or corn was extracted by adding 100 ml chloroform to each culture flask,then shaken for 15 minutes on a wirst-action shaker. After phase separation the chloroform layer was removed and the extraction repeated with additional 100 ml chloroform. Combined extracts were dehydrated over granular anhydrous sodium sulphate and evaporated to dryness at 60ºC in a water bath with liquid nitrogen. Residues were dissolved in 1 ml of water : methnol : acetontril (54 : 29 : 17, v/v/v) and analysis. The total aflatoxins content in liquid medium and rumen content were determined according to AOAC (2006) method using monoclonal antibody columns for total AFs (VICAM Science Technology, Watertown, MA, USA). AFs identification was performed by a modification of the HPLC-Afla test procedure Agillent 1200 Series USA. HPLC equipment with two pumps, column C18, Lichrospher 100 RP-18, (5 µm x 25 cm) was used. The mobile phase consisted of water:methnol :acetontril (54 : 29 : 17, v/v/v) at flow rate of 1 ml / minute. The excitation and emission wavelengths for all AFs were 362 and 460nm (Flourcenses detector), respectively.

***In vitro* dry and organic matter disappearance of corn:**

In vitro dry matter disappearance (IVDMD) and *in vitro* organic matter disappearance (IVOMD) of corn (corn control, *A. flavus* only; *A. flavus* + commercial probiotics; *A. flavus* + *Bacillus megaterium*; *A. flavus* + commercial probiotics + *Bacillus megaterium*) byramrumen fluid were carried out and determined according to Tilley and Terry (1963) and modification suggested by Marten and Barnes, (1979). The rumen fluid were collected from 3 adult rams were fed on clover hay for 20 days.

Statistical analysis:

Data of the experiments were statistically analyzed using the General Linear Model Program of SAS (1996). Significant differences between treatments means were tested by Duncan'S Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

1- Inhibition of *A. flavus* growth by bacterial strains:

L.plantarum (ATCC 14917), *L. curvatus* (ATCC 1136), *B. megaterum* (A.F.10), *B.subtilis* (A.F.12) and commercial probiotics (Table 1) were able to inhibit the growth of *A. flavus* on solid medium (Table 2). The best inhibition was occurred by *B. megaterium* followed by *L. plantarium*. These results agree with results of Corsetti*et al.,* (1998) and Coloretti*et al*., (2007), who reported that lactic acid bacteria (LAB) inhibit mold growth and mycotoxin production. Also, Gomah*et al*., (2009) who found that growth of *A. flavus* was slightly inhibited with the presence of 3 strains of *Lactobacillus spp*. *Latobacilluscurvatus*HBO2 could inhibit growth of

fungi cultured on doubled-layered agar or in liquid anerobic cultivation medium (Dong-mei*et al*., 2008). Inhibition of mold growth by LAB may be due to their action as bio-preservative organisms. Their preserving effect mainly relates to the formation of lactic acid, acetic acid and hydrogen peroxide; competition of nutrients, and the production of bacteriocins (Lindgren and Dobrogosz, 1990; Karunaratne*et al*., 1990).

Table 2.Inhibitory effect of bacterial strains on the growth of *A. flavus*on solid medium.

Bacterial strains	Inhibition zone (mm)
1- *Lactobacillus plantarum*	++ (8mm)
2-*Lactobacillus curvatus*	++ (7 mm)
3-*Bacillus megaterium*	+++ (12 mm)
4- *Bacillus subtitles*	++ (5 mm)

2-Inhibition aflatoxin B_1 production by *A. flavus* on YES media and corn:

The measured of AFB_1 concentration using HPLC showed that all tested bacteria significantly ($P<0.05$) decreased AFB_1 concentration in YES medium and corn (Table 3). The % of decrease ranged from 91.27 to 100% in YES medium and from 49.96 to 69.81 % in corn. The highest decrease in AFB_1production in corn was occurred by *B. megaterium* (69.81%) followed by commercial propiotics (59.62%). The synergistic effect of *B. megaterium* mixed with commercial probiotic on inhibition of AFB_1 production was observed (Table 4). These results are reasonable to that of Gomah*et al*. (2009) who reported that the amounts of AFB_1produced by *A. flavus* in the presence of *Lactobacilli* (5 strain) were reduced by 96.3 to 98.3% compared with control after 10 and 20 days of incubation, respectively. Also, they found that production of AFB_1 by *A. parasiticius* was almost completely inhibited (98.8 to 99.99%) by all the investigated *Lactobacilli*. Also, Farzaneh*et al*. (2012) reported that *Bacillussubtilis* could considerably remediate AFB_1 from nutrient broth culture and pistachio nut by 85.66% and 95% respectively. Moreover, Qing Kong*et al*., (2014) reported that AFs accumulation in potato dextrose broth liquid medium and liquid minimal medium was almost totally (more than 98 %) inhibited by co-cultivation with *Bacillusmegaterium*. Reduction of AFs production in liquid media, milk and intestine by LAB may be due to adsorb of AFs by LAB. Bacterial cell wall binds the toxin with non-covalent weak bonds accompanied with some electrostatic attraction through lactinine like protein, polysaccharides and peptidoglucan (Gratz*et al*., 2005).

Table 3.Inhibition of aflatoxin B_1production of *A. flavus* by commercial probiotic and bacteria.

Treatments	Aflatoxin B_1concentration (ppm)	Inhibition of aflatoxin B_1production (%)*
Yeast extract sucrose medium (YES)		
1- Control (*A. flavus* only)	31.50 ± 1.25 a	0.0
2- A. flavus + commercial probiotic	2.75 ± 0.25 b	91.27
3- A. flavus + *Lactobacillus plantarum*	0.0 ± 0.0 c	100
4- A. flavus+*Lactobacillus curvatus*	0.0 ± 0.0 c	100
5- A. flavus+*Bacillus megaterium*	0.0 ± 0.0 c	100
6- A. flavus+ *Bacillus subtitles*	0.0 ± 0.0 c	100
Corn (Zea maize)		
1-Control (*A. flavus* only)	13.25 ± 0.25 a	0.0
2- A. flavus + commercial probiotic	5.35 ± 0.23 e	59.62
3- A. flavus + *Lactobacillus plantarum*	5.63 ± 0.13 d	57.51
4- A. flavus+*Lactobacillus curvatus*	6.63 ± 0.13 b	49.96
5- A. flavus+*Bacillus megaterium*	4.0 ± 0.0 f	69.81
6- A. flavus+ *Bacillus subtitles*	6.25 ± 0.25 c	52.83

***Inhibition of aflatoxin B_1 production (%) =aflatoxin B_1 concentration of control (*A. flavus* only) -aflatoxinB_1concentration of *A. flavus* treated with bacterial culture /aflatoxin B_1 concentration of control x 100.**

a,b,c,d,e,fMeans in the some row bearing different letters differ significantly ($p<0.05$) n=3.

3. *In vitro* dry and organic matter disappearance of corn:

In vitro dry matter disappearance (IVDMD) and *in vitro* organic matter disappearance (IVOMD) were significantly (P<0.05) decreased in control (fungus only). These results agreed with those reported by Westlake*et al*. (1989), who found reduction in digestion of alfalfa hay contaminated with AFs by ovine rumen fluid (*in vitro*).They suggested that microbial activity was partially inhibited.

Addition of commercial probitoics, *B. megaterium* or mixed of them significantly (P<0.05) increased IVDMD and IVOMD of corn. The highest value of IVDMD (80.49%) and IVOMD (81.28%) were found in mixed commercial probiotics + *B. megaterium*in compared to 53.03% and 81.28%, respectively of control (fungus only).Increasing of IVDMD and IVOMD of corn by addition may be due to decreasing of aflatoxin B_1 concentration in corn (Table 3) and rumen fluid (Table 5)...

Table 4.Synergistic effect of mixed commercial probiotics + *B.megaterium* on inhibition of aflatoxin B_1 production in corn.

Items	Aflatoxin B_1concentration (ppm)	Inhibition of aflatoxin B_1 production (%)*
1- Control (fungus only)	a 13.95 ± 0.23	0.0
2- Fungs + 10 g commercial probiotics.	b 5.65 ± 0.23	59.50
3- Fungs + 10 ml *Bacillus megaterum*	c 3.86 ± 0.21	72.33
4-Fungs + 10 g commercial probiotics + 10 ml *Bacillus megaterium*	d 2.98 ± 0.23	78.64

***Inhibition of aflatoxin B_1 production (%)= aflatoxin B_1 concentration of control (*A. flavus* only) -aflatoxinB_1concentration of *A. flavus* treated with bacterial culture /aflatoxin B_1 concentration of control x 100.**

a,b,c,dMeans in the some row bearing different letters differ significantly (p<0.05)n=3.

Table 5. Effect of mixture of bacteria on dry and organic matter disappearance of corn (*in vitro*) and aflatoxin B_1 content in rumen fluids.

Items	*In vitro* dry matter disappearance (%)	*In vitro* organic matter disappearance (%)	Aflatoxin B_1 content in rumen fluid (ppm)
1- Control (fungus only)	c 53.03 ± 2.64	c 64.22 ± 2.25	a 7.685 ± 188
2- Fungs + 10 g commercial probiotics.	b 72.75 ± 0.94	b 73.01 ± 0.6	b 2.630 ± 190
3- Fungs + 10ml *Bacillus megaterium*	ab 76.71 ± 0.98	a 78.69 ± 0.99	c 1.125 ± 375
4- Fungs + 10 g commercialprobiotics + 10 ml *Bacillus megaterium*	a 80.49 ± 1.39	a 81.28 ± 1.45	d ٠.940 ± 60

a,b,c,dMeans in the some row bearing different letters differ significantly (p<0.05)n=3.

CONCLUSION

The results of the present study showed that all bacteria (*Lactobacillus plantarum*, *Lactobacillus curvatus*, *Bacillus megaterium*, *Bacillus subtitles*) and commercial probiotic have higher ability on inhibition of*A. flavus* growth and its AFB_1 production. The best treatment was *B. megaterium*and commercial probiotics in inhibition ofaflatoxin B_1 production on corn and improving its digestion *in vitro*. Future studies may test the inoculation of agriculture crops by these bacteria at the field harvest and post-harvest stages to reduce aflatoxin in grains, and also to extend the shelf-life of food and feedstuffs.

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تثبيط انتاج الافلاتوكسين ب١ بواسطة البكتريا

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[١] المركز الاقليمى للأغذية والأعلاف – مركز البحوث الزراعية – وزارة الزراعة – الجيزة – مصر.

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تلوث الأغذية بالافلاتوكسين من المشاكل الخطيرة فى العالم. الافلاتوكسينB1 ناتج ثانوى لفطر الاسبرجلس فلافس وله سمية وتاثير سرطانى مرتفع. تقليل تلوث المنتجات الغذائية والأعلاف امكن انجازه بواسطة الكائنات الحية الدقيقة. فى هذه الدراسة تم استخدام بكتريا لاكتوباسلس بلانتاروم ، لاكتوباسلس كورفيتس ، باسلس ميجاتيريوم ، باسلس سائتلس و بروبيوتك تجارى لوقف انتاج الافلاتوكسين ب١ على بيئة مستخلص الخميرة والسكر (YES) والذرة. ثبطت كل سلالات البكتريا انتاج الافلاتوكسين B1من فطر الاسبرجلس فلافس على بيئة مستخلص الخميرة والسكر والذرة. كانت بكتريا الباسلس ميجاتيريوم الافضل تاثيرا حيث ثبطت نمو الفطر وكان قطر المنطقة الخالية من النمو ١٢ ملم كما خفضت انتاج الافلاتوكسينB1فى بيئة (YES) والذرة والتى تم قياسها بواسطة التحليل الكروماتوجرافى السائل (HPLC) ١٠٠% . انتاج الافلاتوكسين B1على الذرة والمقدر باستخدام HPLC انخفض بواسطة الباسلس ميجاتيريوم ٨١.٦٩% تلاه بروبيوتك التجارى (٦٢.٥٩%). وجد تاثير تعاونى بين بروبيوتك التجارى والباسلس ميجاتيريوم فى تقليل انتاج الافلاتوكسين B1فى الذرة وكذلك تحسين هضمه معمليا.