Effect of some Strains of Probiotic Bacteria against Toxicity Induced by Aflatoxins in vivo

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Abstract: Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds produced by some species of Aspergillus, especially A. flavus and A. parasiticus. This study was conducted investigate the effect of some strains of probiotic bacteria against toxicity induced by contaminated diet with aflatoxins in male rats. Animals were divided into 6 equal groups each group contains 7 rats. The first group received a basal diet and served as negative control, the second group received basal diet supplemented with strain 1 of probiotic bacteria (Bifidobacterium bifidum), the third group received basal diet supplemented with strain 2 of probiotic bacteria (Lactobacillus acidophilus), the fourth group received basal diet supplemented with 1.34ppm aflatoxins contaminated peanut as positive control group. The other two groups received basal diet supplemented with 1.34ppm aflatoxins contaminated peanut plus strain 1and strain 2 probiotic bacteria for 6 weeks. Results revealed that positive control gave a very significant increased in alanine aminotransferase (ALT), aspartate aminotransferase (AST), Alkaline Phosphatase (ALP) activities, creatinine and urea; while decreased total protein (TP), albumin and globulin indicating the toxicity of aflatoxin on both liver and kidney functions. However probiotic strains supplemented to aflatoxins treated group revealed a significantly alleviated TP, albumin and globulin depletion in serum with an elevation of ALT, AST, ALP, creatinine and urea; while decreased total protein (TP), albumin and globulin indicating the toxicity of aflatoxin on both liver and kidney functions. The study revealed also that probiotics can also provide benefits by modulating immune functions.

Key words. Mycotoxin, Aflatoxin, Peanut, Toxiciy, Probiotic bacteria

1. Introduction:

Aflatoxins (AFs) are highly toxic secondary metabolites produced by the species of Aspergillus, especially A. flavus and A. parasiticus. These fungi can grow on a wide variety of foods and feeds under favorable temperature and humidity. Contamination by aflatoxins can take place at any point along the food chain from the field, harvest, handling, shipment and storage (Giray et al., 2007).

Aflatoxins (AFS) have been found to contaminate a wide variety of important agricultural products world-wide such as corn, wheat, rice, spices, dried fruits, and nuts. These compounds can enter the food chain mainly by ingestion through the dietary channel of humans and animals (Aycicek et al., 2005). Concerns related to the negative health impacts of AFs have lead to the investigation of strategies to prevent their formation in foods, as well as, to eliminate, inactivate or reduce the bioavailability of these toxins in contaminated products. Techniques to eliminate, inactivate or reduce the bioavailability of AFs include physical, chemical, and biological methods. These processes have at least two drawbacks; high cost of removing and disposing of the contaminated materials and difficulty achieving complete removal contaminated materials without wasting significant portions of uncontaminated product (Méndez-Albores et al., 2007). Limitations such as loss of product nutritional and sensory qualities, as well as, the expensive equipment required for these techniques have encouraged the recent emphasis on biological methods (Teniola et al., 2005).

Lactic acid bacteria (LAB) and bifidobacteria, due in large part to their generally recognized as safe (GRAS) status and use as probiotics, are of particular interest for reducing the bioavailability of AFs. A number of studies have
screened these microorganisms for its ability to bind to AFs and have reported a wide range of genus, species and strain specific binding capacities. Most of previous studies focused on the ex vivo studies but little studies focused on in vivo studies (Hwang et al., 2005; Zinedine et al., 2005; Shahin, 2007; Dalié, 2010).

The whole concept of probiotics is not new, and in fact they have been consumed by human beings in the form of fermented foods, for thousands of years (Kopp-Hoolihan, 2001). Their health benefit has also been long known, in early ages being reported that fermented milk could cure some disorders of the digestive system (Lourens-Hattingh & Viljoen, 2001). Various species of genera Lactobacillus and Bifidobacterium mainly and some other species of micro-organisms have been used as probiotics over the years (Boyle & Tang, 2006). Different strains of Lactobacillus acidophilus and Bifidobacterium bifidum could be considered as the main microbial species that have use as probiotics (Shahin, 2007; Ranadheera et al., 2010). This study aimed to investigate effect of two strains of probiotic bacteria (Bifidobacterium bifidum, and Lactobacillus acidophilus) against toxicity induced by aflatoxins in vivo.

2. Materials and methods

Peanut Samples

Six kilograms of peanut were obtained from Egyptian local market.

Chemicals

Chemicals used in this study were obtained from Sigma Chemical Company (St. Louis, USA).

Media

MRS Broth and MRS Agar were obtained from Oxoid Ltd., Wade Road, Basingstoke, U.K.

Diagnostic Kits

Commercial kits were purchased from Bio Merieux Company (L’Etoile /France) and from Eagle Diagnostics (Dollas, TX, USA).

Probiotic Bacteria

Two strains of probiotic bacteria were used in this study. One of them was obtained from local market and the other has been prepared in vitro.

1- Strain 1 Bifidobacterium bifidum was obtained from Chr. Hansen’s Lab, Denmark. The Bifidobacterium bifidum strain proved to have probiotic properties.

2-Strain 2 (Lactobacillus acidophilus) as Pharmaceutical product manufactured by Ramada (The tenth of Ramadan) CO. 6 of October city ARE (Arab Republic of Egypt) under license of Axcan pharma .S.A. France as a powder.

Animals

Forty two male adult Albino rats (Sprague-Dowley strain) with an average weight 130 ± 10g were obtained from animal house of NRC. Rats were divided into 6 groups (each group 7 rats) and housed in galvanized metal cages. Food and water were supplied ad libtum for 6 weeks. All rats were adapted for three days on the control diet before the beginning of the experiment.

Detection of Aflatoxins

Aflatoxins were detected in peanut sample according to A.O.A.C (1995).

Activation of Tested Strains

Bifidobacterium bifidum was enumerated according to DeMan, et al., (1960) using modified MRS Broth (Oxoid) supplemented with 0.05% L-cysteine HCL (Merck, Germany). Lactobacillus acidophilus was activated in MRS Broth both and anaerobically incubated at 37°C for 24h.

Preparation of Bacterial Strains

Strain1 (Bifidobacteria) was prepared at Food Toxicology and Contaminants, NRC in vitro as follow: 5.0 ml of the activated tested bacteria was added to 500 ml of modified MRS Broth. After that it was incubated at the optimic temperature (37 °C under anaerobic conditions) for 24 hrs then it was cinterifugated at (3000 x g, 4°C, 20 min) to harvest the cells. Dehydration was obtained by addition 50 g of defatted soy protein (soy protein without fat) to cells in big Petri dishes and the cells were incubated under vacuum incubator at 40°C overnight until it seemed like as thin slice or skins. The viability of the cells was tested on MRS agar plates then, the strain was chopped and made as a powder containing 10⁹ of bacteria/g.

The strain 2 (Lactobacillus acidophilus) powder was obtained as Pharmaceutical product containing 10⁹ of bacteria/g. The bucket contains 6 sachets.

Experimental Animals

Diet Preparation
Basal diet was prepared according to the method described by Campbell, (1963) on diet bases: Protein (12%), fat (10%), salt mixture (4%) vitamin mixture (1%), Choline chloride (0.25%), and cellulose (5%) corn starch (up to 100). The vitamin mixture was prepared according to Campbell, (1963). The salt mixture was prepared according to Hegsted et al., (1941).

Experimental Design
The forty two rats were divided to 6 equal groups as following:
- Group 1 (G1): fed on basal diet (negative control);
- Group 2 (G2): fed on basal diet + strain 1 of probiotic bacteria (Bifidobacterium bifidum).
- Group 3 (G3): fed on basal diet + strain 2 of probiotic bacteria (L. acidophilus).
- Group 4 (G4): fed on 10% natural contaminated peanut with aflatoxins (Positive control).
- Group 5 (G5): fed on 10% contaminated peanut with aflatoxin + strain 1 of probiotic bacteria;
- Group 6 (G6): fed on 10% contaminated food of aflatoxin + strain 2 of probiotic bacteria.

Biochemical Analyses
At the end of the experiment rats were fasted overnight (about 12 hrs) and anesthetized with diethyl ether. Blood samples were collected in clean dry centrifuge tubes from hepatic portal vein. All blood samples were centrifuged for 15 minutes at 3000 rpm to separate the serum. Serum was kept frozen at (-20°C) till analysis (3-5 days). According to (Jacobs et al., 2001).The toxicity of aflatoxins and the protective effect of Probiotic bacteria against aflatoxins toxicity were evaluated by determination of serum ALT and AST activities according to Henry (1974) and Yound (1975), respectively. Enzymatic calorimetric determination of serum alkaline Phosphatase was carried out according to Belfield and Goldberg (1971) as liver function tests.

Determination of Serum Total Protein (T.P) according to (Gornal et al.,1949) and Serum albumin was determined as g/dl according to the method described by Weiss man et al., (1950).

Serum Globulin was calculated as g/dl according to Chary and Sharma, (2004).

Serum A/G Ratio was calculated according to Sirvastava et al., (2002).

Statistical Analysis
Statistical analysis was performed by using computer program COSTATE and compared with each other using the suitable tests. We used one way ANOVA ((Armitage and berry (1987)

Results are reported as 1-mean ± SD
2- P value differences with p ≤ 0.05 were considered to be significant p<0.05 very significant

3. Results and Discussion:
Levels of aflatoxins in peanut and contaminated diet
As shown in table (1); levels of aflatoxins B1, B2, G1, G2 and total aflatoxins were 3.3±0.8, 1.0±0.2, 7.0±1.7, 2.1±0.5 and 13±3.3 µg/kg peanut respectively. The levels of aflatoxins in basal diet supplemented with 10% contaminated peanut were 0.33, 0.1, 0.02, 0.7, 0.21 and 1.34 µg/kg for aflatoxin B1, B2, G1, G2 and total aflatoxins respectively. These results agree with Ayesh and Ismail, (2001) who screened the toxigenic fungi and aflatoxins production in different variety of peanut (Early Bunch, Gregory, Romy and NC) before and during the different processing stages as well as during storage and Sultan (2004) who reported that the aflatoxins in naturally contaminated peanut seed reached to 26.7ppb.

The knowledge that mycotoxins can have serious effects on humans and animals has led many countries to establish maximum tolerated level (MTL) on mycotoxins in foodstuffs and feedstuffs in the last decades to safeguard the health of humans, as well as the economical interests of producers and
traders. Currently, worldwide range of limits for AFB1 and total AF (AFT) are 1-20 ng/g and 0–35 ng/g, respectively (FAO, 2004).

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Amounts (µg/Kg)</th>
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<tbody>
<tr>
<td>AFB1</td>
<td>3.3±0.8</td>
</tr>
<tr>
<td>AFB2</td>
<td>1.0±0.2</td>
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<tr>
<td>AFG1</td>
<td>7.0±1.7</td>
</tr>
<tr>
<td>AFG2</td>
<td>2.1±0.5</td>
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<tr>
<td>T AF</td>
<td>13.4±3.3</td>
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Table 1: Levels (µg/Kg) of Aflatoxins in Peanut

Effect of probiotic bacteria on body weight gain, food intake and feed efficiency ratio

As shown in Fig 1, the group received contaminated peanut with aflatoxins showed significantly lower in BWG, FI and FER (p<0.05) compared with basal diet which may be due to the loss of animals appetite caused by aflatoxin. Similar results were obtained by Parlat et al., (1999) who found that BWG and feed conversion rate (FCR) were decreased significantly by AFB1 treatment compared with control and Denli et al., (2003) who reported that aflatoxin B1 (AFB1) caused non significant reduction in Body Weight Gain (BWG) and (FCR) by 9.3 and 7.6 % respectively.

The decreased in BWG, FI and FER were significantly (P ≤ 0.05) improved (by probiotic bacteria supplemented to aflatoxin treated group. In addition BWG in the group received probiotic bacteria strain2 (lactobacillus acidophilus) was significantly (P ≤ 0.05) higher compared with negative control and FI and FER were around negative control. These results indicated the health benefit and the effect of probiotic bacteria against toxicity induced by aflatoxins. This occurred as a result of decreased uptake of toxins caused by Bifidobacteria which lead to increasing FER and BWG (Solga, 2003).
Effect of probiotic bacteria on liver functions in rats fed aflatoxins contaminated diet

The results in Fig 2 (A,B,C) showed that aflatoxins treatment caused very significant (P < 0.05) increased on serum liver function enzymes ALT, AST and ALP. The affected liver functions by aflatoxins were achieved by Zohair (1996), Denli et al., (2003) and Kermanshahi, et al., (2007) who demonstrated that feeding aflatoxin B1 (AFB1) may have some adverse effects on the liver and brain of broilers. Probiotic strains Bifidobacterium bifidum and lactobacillus acidophilus supplemented to aflatoxins treated group showed a significant (P < 0.05) improved in liver functions. It was also noticed that lactobacillus acidophilus is better than Bifidobacterium bifidum strain. This occurred as a result of the ability of microorganisms to bind aflatoxins and have reported a wide range of genus, species and strain specific binding capacities (Peltonen et al., 2000; Peltonen et al., 2001). In addition; Peltonen et al., (2000) assessed the ability of six probiotic bacteria to bind a common food carcinogen, aflatoxin B1 in vitro. The studied strains included Lactobacillus strains and one Bifidobacterium strain. The aflatoxin-binding capacity of the strains was found to range from 5.8 to 31.3%. Although the extent of binding varies depending on the bacterial strain used, the data may explain some of the antimutagenic and anticarcinogenic effects of probiotic microorganisms.

In vivo study, EL-Nezami et al., (2006) concluded that probiotic supplement reduced the biologically effective dose of aflatoxin exposure and may thereby offer an effective dietary approach to decrease the risk of liver cancer. Also Gratz et al., (2007) found that probiotics, especially GG are able to bind AFB1 under in vivo conditions in rats and intestinal cells.
Effect of probiotic bacteria on total protein, albumin, globulin and A/G ratio in rats fed aflatoxins contaminated diet

The results in Fig 3 indicated that the group received contaminated diet with aflatoxins showed high significant (p<0.05) decreased in total protein, albumin, globulin and A/G ratio. This agrees with those reported by Zohair (1996) in rats and Matri (2001) in Japanese quail birds.

![serum total protein levels](image1)

![serum albumin levels](image2)

![serum globulin levels](image3)

![A/G Ratio levels](image4)

The decreased in total protein, were improved by probiotic strains *Bifidobacterium bifidum* and *lactobacillus acidophilus* supplemented to aflatoxins treated group compared to aflatoxin group On the other hand albumin showed significant (p ≤ 0.05) improvement indicating the capability of probiotic bacteria to reduce the toxicity induced by aflatoxins.

**Fig.3:** Effect of probiotic bacteria on total protein, albumin, globulin and A/G ratio in rats fed aflatoxins contaminated diet
Effect of probiotic bacteria on Kidney functions in rats fed aflatoxins contaminated diet

Fig 4 showed that the group received contaminated diet with aflatoxin showed very significantly (p<0.05) higher in urea and creatinine levels, as compared to healthy rats fed on basal diet indicated the toxicity of aflatoxin on kidney functions. These results were in coincide with those reported by Zohair (1996) in treated rats and those of Matri, (2001) in Japanese quail birds received contaminated feed with aflatoxin and showing significant higher (p<0.05) in serum total cholesterol, creatinine and urea. On the other hand the intakes of both probiotic bacteria strains significantly (p ≤0.05) alleviated the elevation of urea level in aflatoxins treated rats. This result showed the detoxification activity of probiotic strains.

The probiotic with AFB1 bound to their surfaces likely to adhere to the intestinal wall and prolong exposure to dietary aflatoxin. Hence, specific probiotics may be potent and safe means to reduce absorption (Gratz et al., 2006). In addition the protective effects of probiotic bacteria against aflatoxin B1 induced intestinal and systemic toxicity via binding and reducing its transport in different tested systems (Gratz, 2007). The role of probiotic bacteria in improving the immunity may be also explained the detoxification activity of probiotic bacteria. There is now substantial evidence that probiotics can provide benefits by modulating immune functions. In animal models, probiotic supplementation is able to provide protection from spontaneous and chemically induced colitis by down regulating inflammatory cytokines or inducing regulatory mechanisms in a strain-specific manner (Borchers et al., 2009).

Fig 4: Effect of probiotic bacteria on total Kidney functions in rats fed aflatoxins contaminated diet

Effect of probiotic bacteria on organs weight of rats fed on aflatoxin contaminated diet

It could be noticed from Fig 5 that the group received contaminated diet with aflatoxins showed significantly (p ≤0.05) increased in organs weight (heart, kidney) and very significant (p<0.05) increased in liver weight, as compared to basal diet group. The intake of probiotic bacteria showed significantly (p ≤0.05) lower and improved organs weight in aflatoxins treated rats as compared to positive control. Gratz et al., (2006) suggested that by increasing the excretion of orally dosed aflatoxin via the fecal route, probiotic treatment prevents weight loss and reduces hepatotoxic effects caused by a high dose of AFB.
Results of histopathology

Kidneys of rat from group1 which was fed on basal diet for 6 weeks revealed the normal histological structure of renal parenchyma (photo 1). However, kidneys of rat from group 4 which was fed on contaminated diet with aflatoxin (10%/Kg diet) revealed marked dilatation and congestion of renal blood vessels and vacuolation of epithelial lining renal tubules (photo 2). Examined sections for groups 5: rats received aflatoxins + strain 1(Bifidobacterium bifidum) and group 6: aflatoxins +strain2 (Lactobacillus acidophilus showed no histopathological changes (photos 3, 4).

Liver of rat from group 1 which was fed on basal diet for 6 weeks revealed the normal histological structure of hepatic lobule (Photo 5). However, liver of rat from group 4 which was fed on contaminated diet with (10%/Kg diet) aflatoxin showed vacuolar degeneration of hepatocytes and fibrosis in the portal triad (Photo6). Some examined sections for group 5 which was fed on 10% contaminated diet with aflatoxin + strain 1(Bifidobacteria) showed no Histopathological changes except vacuolation of sporadic hepatocytes (Photo7), and other sections revealed no Histopathological changes (Photo8). Moreover, liver

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Fig. 5: Effect of probiotic bacteria on organs weight of rats fed on aflatoxin contaminated diet.
of rat for group 6 which were fed on 10% contaminated diet with aflatoxin + strain 2 (L. acidophilus) showed no histopathological changes except dilatation and congestion of central vein and hepatic sinusoids (Photo 9). Other sections from the same group revealed no histopathological changes (Photo 10).

The results of histopathology obtained indicate the toxicity of aflatoxins on liver and kidney, these results walk in the same line with numerous animal studies which have shown that the liver is the main target organ and therefore the main symptoms of aflatoxin exposure in domestic laboratory animals are hepatic injuries (Robins and Richard, 1992; IARC, 1993). In addition Matri, (2001) reported that sever histopathological changes was observed in the liver, kidney, heart, ovary and oviduct during aflatoxicosis. Also these results agree with Yener et al, (2009) who reported that the livers of the AF-treated group were slightly pale, enlarged and grayish mottled in appearance. However addition of probiotic strains to aflatoxin treated rats showed improved in the liver sections and showed no histopathological changes in kidneys as negative control. These results showed the effective role of probiotic bacteria especially the strains Bifidobacteria and lactobacillus acidophilus against toxicity induced by aflatoxins. These results are agree with Bekhatro, (2008) who reported that liver of rat fed on B. bifidum 29521 showed no histopathological changes except minute vacuoles in the cytoplasm of some hepatocytes.

In conclusion: the previous results indicated the protective action of probiotic strains Bifidobacteria and lactobacillus acidophilus as a potential protective agent against aflatoxin toxicity as well as their beneficial health effects and may thereby offer an effective dietary approach to decrease the risk cancer as a result of its ability of probiotic strains to bind with aflatoxins, inhibiting their absorption and protected against both memberane and DNA damage. Probiotics can also provide benefits by modulating immune functions. The data may be explained some of the antimutagenic and anticarcinogenic effects of probiotics microorganism.

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Photo (1): Kidney of rat from G1 showing the normal histological structure of renal parenchyma.

Photo (2): Kidney of rat from G4 showing marked dilatation and congestion of renal blood vessel.

Photo (3): Kidney of rat from G5 showing no histopathological changes.

Photo (4): Kidney of rat from G6 showing no histopathological changes.
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