J. Agric. Sci. Mansoura Univ., 33 (7): 5173 - 5187, 2008

FACTORS AFFECTING LACTIC ACID BACTERIA ADSORPTION OF AFLATOXIN AND THE EFFECT OF ADSORBED TOXIN ON RATS' LIVER TISSUES

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ABSTRACT

Aflatoxin adsorption property of lactic acid bacteria could be used as a detoxification method which is most appropriate for food and dairy products. Seven strains of lactic acid bacteria (*Lactobacillus casei, Lactobacillus gasseri, Lactobacillus reuteri, Lactobacillus bulgaricus, Lactobacillus acidophilus*, *Streptococcus thermophilus, Bifidobacterium bifidium*) plus two mixed commercial cultures FD–DVS YCX11 50U (*Lactobacillus bulgaricus+ Streptococcus thermophilus) and* FD–DVS ABT2 50U *(Lactobacillus acidophilus+ Streptococcus thermophilus* +Bifidobacterium bifidum*)* were tested for their AFB_1 and AFM_1 adsorption rate, Factors affecting such adsorption were studied. The bioavailability of the adsorbed toxin was determined by feeding rats with L. casei-AFB_1, L. casei-AFM_1 complexes, and both toxins and rats liver tissues were examined. Adsorption reaction was carried out in 1 ml of phosphate buffer saline containing 1µg of the toxin and about $1.7x10^{11}$ CFU of bacteria at 37° C for 2 hrs and pH of 7.3. out of the tested strains,L. casei showed the highest toxin removal rate of 34.1% and 27.7% for AFB_1 and AFM_1, respectively. These rates were increased by reducing the toxin into 0.5µg /ml, lowering the reaction pH into acidic pH and using acid or heat killed cells. Moreover, the rate was increased by adapting L. casei to toxin adsorption by repeated exposure to toxin, the rate increased from 34.1% to 50% of AFB_1. Toxin concentration higher than 0.5µg / ml reduced the adsorption and caused changes in cell morphology. Aflatoxin bound by bacteria showed no toxicity effect on rats. Liver tissues of rats fed on the toxin complex were normal structure as compared to tissues of rats fed on free toxins which showed macroviscular fatty change, hydropic degeneration and congested hepatic sinusoids . the toxin complex was not absorbed since it did not adhere to intestinal wall. Yoghurt and sweet cultured milk were processed from AFM_1 contaminated milk using L. casei culture.

INTRODUCTION

Aflatoxins are group of mycotoxins with mutagenic, carcinogenic, and immune suppressive properties. They have been classified by International Agency for Research in Cancer (IARC) as a class one human carcinogen (**15**). The toxin contamination is widespread and reaching humans or animals through their foods or by inhalation. When absorbed into blood from small intestine, the toxin accumulates in various tissues of the body including the tissues of liver and respiratory, renal and gastrointestinal nervous, reproductive and immune system. Of all tissues, liver contains the highest concentration, about 10 fold higher than in muscles (**18**). AFB1 appeared in liver proteins after 6 h from injection, and hepatic enzymes are released after 24h. AFB1 was found in nucleus of liver cells, interacting with DNA, inhibiting RNA production (**4**) and producing liver tumors which are hepatocellular carcinoma (**3**). Moreover the AFB1 ingestion or inhalation by animal farm

caused the decrease in milk & egg production, and is excreted in milk, eggs, and animal tissues (**10**).

A variety of detoxification methods for human foods and animal feeds have been reported. These included Physical methods (bentonite, hydrated sodium calcium aluminosilicate, and clay adsorbents) Chemical methods (5% NaOCl, 10% Chlorine gas, ammonia, and solvent extraction), phytochemicals (iso-thiocyanate, flavonoids, allicin, and chlorophyllin), and biological methods (Flavobacterium aurantiacum), However, a practical, large scale, cost effective and safe method for a complete detoxification of aflatoxin – containing human food or animal feeds are currently not available (**9**). The problem becomes more difficult with human foods since the safe treatments or additives that could be permitted are difficult to find. For example, milk and dairy products such as ripened cheese are usually contaminated with aflatoxin M_1, and sometimes with aflatoxin B_1, cannot accept any of the above additive or treatments.

Probiotic bacteria, microorganisms that confer health benefits when consumed by humans, are mostly lactic acid bacteria (LAB) and their safety has been proven by their safe use for ages. Beside their health benefits, probiotics protect against food mutagens such as heterocyclic amines, nitroso-compounds and aflatoxin (**14**). Certain strains from LAB have been reported to adsorb aflatoxins B_1, B_2, G_1, G_2, M_1, M_2 and Zearalenone from liquid media, milk and from the intestine forming a stable complex (**11**, **10**, **20**). Viable and heat or acid killed bacteria bind the toxin and the rate of binding depends on the microorganism and the strain and is linear with toxin, and bacterial concentration.

The adsorption is a physical process, bacterial cell wall binds the toxin with non – covalent weak bonds accompanied with some electrostatic attraction through lactinine like protein, polysaccharides and peptidoglycan (**13**).Toxin binding is a fast process, its optimum temperature and pH were 35 - 37°C, 6 – 7.5 respectively (**1**). The adsorption rates varied from 5.6% by Lactobacillus lactis to 88% in vitro and 92% in vivo (chicks) by Lactobacillus rhamnosus GG (**10**, **21**). L. rhamnosus GG and L. rhamnosus Lc705 removed AFM_1, from skim and whole milk with lower percentages than from phosphate buffer medium, with a range from 18.8 to 69.6% (**20**).Number of treatments affects the bacterial adsorption of the toxin. Treating the bacteria with 1M of HCl, boiling at 100°C, and autoclaving increased toxin binding and the acid treatment was the most effective. Exposure of the cells to ethanol, U.V. irradiation, sonication and alkali showed either no effect or reduced binding (**9**). Heat as well as acid alter surface properties of bacteria leading to higher toxin adsorption and lower desorption rates.

The complex, though stable, is reversible and extracelluler nature, its stability depends on strain, treatment and the environmental conditions (**21**). Actually, conditions, in duodenum enhanced bacteria to bind the toxin and improved the complex stability, and the toxin is not released back into the duodenum contents. The complex was found to be stable under the luminal conditions for one hour (**1**).The toxin can be removed from the complex partly (~30%) by excessive washing with buffer solution and almost completely

(90%) with organic solvents. However, autoclaving, temperature exposure from 4 to 37°C and pH 2 -10 did not release the toxin (**14**).

Most important properity of this method is that binding the toxin by bacteria reduced their adhesion into the intestinal epithelium, preventing its accumulation in the intestine causing its release out of the body (15). For example, the complex reduced capability of L. rhamnosus GG adhesion from 30 to 5 %. There was a 74% reduction in the uptake of AFB_1 by the intestinal tissue in the presence of L. rhamnosus GG. In vitro LAB reduced AFB_1 by 54% in the soluble fraction of luminal fluid in one minute. Therefore this properity allowed the detoxification without the need for the removal of the bacterial-toxin complex from the food which is an impractical step. Actually, the toxin changed bacterial cells morphology and this might lead to a change in adhesion sites. (15).

Therefore, probiotics beside their immune modulating effect are good prospect for physical detoxification of foods. Actually they are regularly used in food processing and if they are not part of the process, heat killed bacteria can do the detoxification without altering the taste or acidity of the food. However, that this method renders the toxin unavailable for absorption in the intestine thus alleviating the toxin harmful effect is yet to be proven. Moreover, the method requires screening the available probiotic bacteria for selecting the proper microorganism and the strain having high adsorption rate that fits processing of a certain food, then studying the factors within the food affecting complex formation. Therefore, research was carried out to:

1. Test number of the available probiotics for their rate of AFB_1 & AFM_1 binding and study the affecting factors.
2. Use of the selected probiotic bacteria for manufacturing yogurt & sweet cultured milk from AFM_1 contaminated milk.
3. Determine the adhesion of AFM – bacterial complex to mucus cells.
4. Study the morphological changes of probiotic on binding the toxin.
5. Determine the bioavailabity of the toxin when adsorbed by bacteria by comparing the effect of feeding rats on pure AFB_1 and AFM_1 as well as their bacterial – toxin complex on rats' liver tissues.

MATERIALS AND METHODS

Bacterial strains:

Lactobacillus casei, Lactobacillus gasseri, Lactobacillus reuteri, Lactobacillus bulgaricus, Lactobacillus acidophilus, *Streptococcus thermophilus, Bifidobacterium bifidium,* were obtained from National Research Center (NRC). Two commercial cultures FD–DVS ABT2 50U *(Lactobacillus acidophilus*+ *Streptococcus thermophilus* +Bifidobacterium) and FD–DVS YCX11 50U *(Lactobacillus bulgaricus*+ *Streptococcus thermophilus)* were from MIFAD Company, Egypt.

The cultures were activated in 11% reconstituted skim milk for several times and the last 3 times were in De Man Regosa and sharp medium at 37˚c except for S. thermophilus & L. bulgaricus were at 40˚c. Cystein was added to Bifidobacterium. Standard plate count on MRS agar was used for

bacterial count (CFU). After activation, maximum counts obtained were L. casei ($1.7x10^{11}$), L. reuteri ($1.1x10^{11}$), L. gasseri ($9.3 x10^{10}$), Lactobacillus bulgaricus $7.9x10^{10}$, Lactobacillus acidophilus $9.1 x 10^{10}$, Streptococcus thermophilus, $7. 2x 10^{10}$, Bifidobacterium bifidium $1.3 x 10^{11}$.the two commercial cultures Y C X 11 50U and A T B 2 50U contained $7.3 X 10^{10}$ and $1.6 X 10^{10}$ respectively.

Phosphate buffer saline:

Phosphate buffer saline solution was the medium for the toxin – bacterial binding reaction. Saline helps the complex formation. One tenth of mole of the buffer was prepared according to Lee, Y.K et al. 2003(16) and the pH was adjusted to 7.3.

Aflatoxins:

Crystalline aflatoxin B_1 and M_1, (AFB_1, AFM_1) were from Sigma Aldrich, St Louis, MO, USA. Stock solutions (1mg/1ml) were prepared in acetonitrile/benzene (98/2). Methanolic working stocks were prepared by evaporating the acetonitrile/benzene solvent at 80°C, and reconstituting the solid in methanol. stocks were stored at 20°C. Aqueous working solutions of various concentrations were prepared by mixing alcoholic solution with phosphate buffer saline (PBS) (1ug/ml). AFB_1 in reaction medium after centrifuging out bacterial-toxin complex was extracted and cleaned up according to CB method (AOAC, 2000) using silica gel column, while sep-pack Vac RC (500mg) C_{18} cartridge was used for AFM_1. The dry film of the toxin was derivatized with trifluoroacetic acid, and derivative were quantitated with HPLC using Agilent Zorbax SB-Aq column and l. akobra cell for past column derivatization.

Toxin adsorption and factors affecting:

AFB_1 and AFM_1 adsorption rates were determined by mixing the bacteria ($1.7x10^{11}$ CFU) in 1 ml of PBS containing 1 µg of the toxin for 2 hrs at 37°C. The bacteria was centrifuged out (3000 rpm), and the toxin in the supernatant was determined and binding rates were calculated. Toxins desorption from AFB_1 or AFM_1 bacterial- complex was determined by washing bacterial complex pellets (formed by $1.7x10^{11}$ CFU plus 1µg toxin) 5 times with 5ml PBS. The rinsing buffer was combined and their contents of the toxin were determined.

The effect of repeated exposure of bacteria to toxin on rate of toxin binding was determined by mixing L. casei ($1.7x10^{11}$ CFU) with the toxin for 2 hrs at 37°C, and then the cells were centrifuged out of the mix. Toxin left in the supernatant was determined to calculate binding percent. The pellets were reactivated in 10 ml skim milk for 36 hrs at 37°C to get $1.7x10^{11}$ CFU/ml , and the activated cells were mixed with the toxin for binding, the above steps were repeated for 7 times.

Viability of heat (100°C for 1 hr) or acid killed (pH 2.37 by 1M Hcl) bacteria was determined by standard plat count (SPC). The effect of mixing L. casei with AFB_1 and AFM_1 on bacteria morphological changes was determined by spreading a smear from the adsorbed mixture over a microscope slide. The film was gram stained and examined with a Leica light microscope.

Bioavailability of toxin bound to bacteria and its effect on rats' liver tissue

To study the bioavailability of toxin after binding with L. casei, 49 rats from NRC animal house were divided into 7 groups each group contained 7 rats. Rats were fed on basal diet for 3 weeks for adaptation before the injection and basal diet continued through the experiment. The toxin solution, bacteria and their complex suspensions in PBS were injected with a special needle into the digestive system of the rat through the mouth for 3 weeks. One ml of the toxin (1µg/ml of PBS) was orally injected daily in groups 1 and 2 given a sum of 21 µg of AFB_1 or AFM_1 for each rat. L. casei – toxin complex (which was prepared from 1 µg toxin with $1.7x10^{11}$ CFU) was injected daily in groups 5 and 6. Lactobacillus casei suspension of $1.7x10^{11}$ CFU in 1 ml PBS was orally injected daily in groups 2 and 3. the seven groups were:

1: AFB_1 solution.
2: AFM_1 solution.
3: L. casei suspension (from NRC)
4: L. casei suspension (a 1:1 mixture of two commercial, MIFAD Co.,)
5: L. casei – AFB_1 complex
6: L. casei – AFM_1 complex
7: control (basal diet contains of wheat 22%, corn 62%, salts mix 3%,vitamins 5% and Soya oil 7%).

At the end of the experiment, rats were slaughtered; their livers were soaked in 10% formalin for 24 hr and dehydrated with ethanol with increasing concentration (70, 80, 90&100%), 30 min for each concentration. The tissues were then placed in paraffin for one day and cut into slices (5-10µm) with a microtome. Slices were spread over glass slides, air dried, stained with Hematoxyline and Eosin stain solution and the tissues morphology were examined by a Leica light microscope equipped with camera.

Bacterial adhesion to epithelial cells:

The effects of toxin binding on bacterial adhesion into columnar epithelial cells of rats' small intestine were tested as follows:

Segments of rats' small intestine (4 cm) held in PBS at 4˚c for 30min then were rinsed 4 times with the buffer. The epithelial cells were scrapped off from the surface of the intestine with the edge of a glass slide and suspended in PBS. Epithelial cells suspension was mixed with either bacteria or bacteria binding the toxin. After incubation for two hours at 37°C a smear from each mix was spread on a glass slide, Gram stained and the adhesion was viewed by a light microscope (**15**).

Removal of AFM_1 from milk:

Reconstituted skim milk 11% was sterilized at 110°C for 30 minute cooled to 40°C spiked with 10 µg AFM/ 10ml of milk and was processed. Yogurt was processed by inoculating the tube with $1X10^{11}$ of L. Casei, incubating at 40°C until coagulation (about 4h), then stored for 36 hr under refrigeration before toxin determination. Sweet cultured milk was processed by inoculating the contaminated milk(10µg aflatoxin/10ml) with $1X10^{11}$ CFU/ml of L. casei & L. acidophilus (1:1, v/v) at 40°C after stirring cooled to 4°C and kept cold for 4 days before toxin determination.

Statistical analysis:

Triplicate samples were analyzed and the results were statistically analyzed by M – stat program and Excel.

RESULT AND DISCUSSION

Since it is important to screen large number of probiotics for their toxin adsorption, numbers of lactic acid bacteria (LAB) have been screened for their rate of AFB_1 and AFM_1 binding. Seven strains from NRC & two commercial cultures were tested and results are in Table (1). L. casei showed significantly ($p > 0.05$) better ability to remove AFB_1 and AFM_1 from PBS(34.1 & 27.7) at 37°C than other strains, followed by L. reuteri (34.1 & 24.82%) and S. thermophilus was the lowest in binding (18% & 13.9% for AFB_1 & AFM_1, respectively). The two commercial cultures YCX11 50U (*Lactobacillus bulgaricus+ Streptococcus thermophilus.*), and ABT2 50U (*Lactobacillus acidophilus+ Streptococcus thermophilus +Bifidobacterium*.) showed intermediate ability for toxin binding. These binding rates were with bacteria cell counts mentioned in Table (1) footnotes. However, using lower cell counts led to lower toxin removal rate. For example, in our experiments, when L. casei count was at $1x10^6$ cfu/ml removed 18.5 and 16.4% from AFB_1 and AFM_1 respectively.

Table (1): Rate of aflatoxin binding by probiotic bacteria:

Strain [2]	Toxin removal % [1,3]	
	AFB1	AFM1
Lactobacillus casei	34.1	27.7
Lactobacillus reuteri	33.00	24.82
Lactobacillus gasseri	29.95	21.98
Lactobacillus bulgaricus	24.91	16.00
Lactobacillus acidophilus	27.01	21.1
Streptococcus thermophilus	18	13.9
Bifidobacterium bifidium	22.52	15.53
Y C X 11 50U [4]	21.98	17.01
A B T 2 50U [5]	26.78	20.98

1. Toxin concentration 1µg/1ml PBS,

2. Bacterial concentrations were Lb. casei ($1.7x10^{11}$), Lb. reuteri ($1.1x10^{11}$) Lb gasseri ($9.3x10^{10}$) Lactobacillus bulgaricus ($7.9x10^{10}$) Lactobacillus acidophilus ($9.1x10^{10}$) Streptococcus thermophilus 7. 2 x 10^{10} Bifidobacterium bifidium $1.3x10^{11}$, Y C X 11 50U 7.3 X 10^{10}, A B T 2 50U 1.6 X 10^{10} CFU

3. Reaction temperature 37˚C for 2 h,

4. *Lactobacillus acidophilus+ Streptococcus thermophilus* +Bifidobacterium bifidum($7.3x10^{10}$).

5. *Lactobacillus bulgaricus+ Streptococcus thermophilus($1.6x10^{10}$).*

However, the concentration of $1x10^6$ is recommended for probiotic products to show health benefits, therefore, the recommended cell load should be modified when probiotic is used for health benefits as well as detoxification. AFM_1 binding by bacteria was less than AFB_1. These binding values were lower than the reported values for other bacteria such as L.

rhamnosus GG (78%) (**14**). Different bacteria as well as their strains vary in their binding ability (**21**), this is why screening to select the proper strain is important. When L. casei, L. reuteri, and L. gasseri grew in a mixture the toxin binding rate of the mix was not enhanced (unreported data). In other words, there was no synergistic effect on strain mixing. Stability of the complex against washing is important for the completion of detoxification; therefore, the stability against PBS washing was determined (Table 2). First washing was the most effective giving significant toxin removal, and from the third washing the complex was almost unaffected. L. casei, which was the highest in binding rate, formed the most stable complex which was not significantly affected by washings. L. reuteri was the second in toxin binding and complex stability after L. casei. Aflatoxin M_1 complex followed the trend of AFB_1 , again L. Casei formed the most stable complex, though lost more toxin on first washing than with AFB_1. Both properties, rate of binding and complex stability would be expected to depend on cell wall composition and number of binding sites and the chemical composition of the toxin. The results pointed out the importance of selecting the strain of highest binding rate.

Table (2): Effect of washing on removal of toxin from bacterial toxin complex:

Bacterial type	Toxin bound after washing, %									
	Aflatoxin B_1					Aflatoxin M_1				
	No. of washes					No. of washes				
	0	1	2	3	4	0	1	2	3	4
L. casei	34.1	33.5	32.8	32.71	32.66	27.7	25.54	23.1	22.99	22.91
L. reuteri	32.8	29.06	28.03	27.65	27.61	25.16	20.92	19.49	19.29	19.19
L. gasseri	30.4	27.48	25.09	24.55	24.38	22.20	18.00	17.58	16.95	16.92
L. acidophilus	27.09	21.20	19.93	19.71	19.68	20.80	15.89	14.20	14.11	14.1

1. **Five ml of phosphate buffer saline was used for each washing.**
2. **Number of cells used were Lb. casei ($1.7x10^{11}$), Lb. reuteri($1.1x10^{11}$) Lb gasseri ($9.3x10^{10}$) , and Lb acidophilus ($9.1X10^{10}$) CFU/ml.**

L. casei, the highest in binding rate, was used to study the factors that affecting its binding rate. Table (3) reports the effect of temperature of mixing L. casei with the toxin on binding ratio.

Table (3): The effect of Temperature of mixing Lb. casei with the toxin on its binding rate:

Temperature of mixing , °C	*Toxin removal, %*	
	Aflatoxin B_1	Aflatoxin M_1
4	18.3	*8.51*
20	24.7	*15.31*
27	29.1	*21.03*
37	34.1	*27.6*
40	*32.9*	*26.01*

1. **Aflatoxin concentration was 1µg / 1ml buffer and mixed with $1.7x10^{11}$CFU.**
2. **Incubation time was 2 hrs.**

Mixing temperature affected the binding rate, low temperature (4˚C) wasn't proper for high rate binding which occurred only at 37˚C. At 40 °C, binding started to decline, this point is important when selecting the step at which the culture is added during food processing, the step should allow a proper time at the 37°C. Reaction period is another factor that affect binding rate, therefore, the effect of different reaction periods on binding rates, and the stability of the complex was studied and results are in Table(4).

Table (4) :Effect of incubation period of Lb. casei with toxin on binding rate and the complex stability:

Incubation Period , hrs.	TOXIN BINDING RATE , %			
	Aflatoxin B_1		Aflatoxin M_1	
	Before washing	After washing	Before washing	After washing
2	34.1	33.92	26.91	26. 4
4	34.6	34.13	26.96	26.52
24	35.6	34.4	27.68	26.99
48	38.2	34.52	29.99	27.89
72	39.3	35.01	30.53	28.01

1. Bacterial concentration $1.7x10^{11}$ CFU/ml.
2.Toxin concentration 1µg/1ml.
3.Washing carried out with 5ml of buffer five times.

Most of aflatoxin B_1 and aflatoxin M_1 binding occurred within 2 hrs, after which binding continued at low rate. After 48 hr, there was a significant difference for both toxins from 2 hrs level. As for the complex stability, 2 hrs reaction was the most stable; there was almost no release of the toxin after 5 buffer washings. On the other hand the increase in binding after 24 hrs, was desorbed by washing, they were less tightly bound. Therefore, 2 or 4 hrs period is good enough for the process. These results favor the use of this method in some dairy processing such as yoghurt or ripened cheese which stays for long during storage.

Table (5) shows the effect of toxin concentration on rate of toxin removal by L. casei from PBS. At toxin concentration of 0.5 µg / ml the toxin was removed by 40.4 and 31.9% for AFB_1 and AFM_1, respectively. Beyond such concentration, the binding rate significantly decreased reaching at 5.0 µg a minimum of 16.5 and 16.9% for AFB_1 and AFM_1, respectively. This change would probably stem from the fact that high concentration of AFB_1 (5 ppm) interfered with wall synthesis causing the inhibition of S. lactis and Flavobacterium growth. Beside growth inhibition, cells developed aberrant morphology forms by enhancing its length with swollen and branched ends and forming along chains (**5, 7**). Our results showed that low adsorption rate at high toxin concentration was accompanied with cell morphological changes. Fig (1) shows the morphology of L. Casei when exposed to 10 µg AFB_1 and AFM_1 for 24, 48, and 72 hrs. The elongation, end branching and long chains appeared after 48 hr of exposure. Changes that caused this aberrant morphology probably interfered with toxin binding sites by limiting their numbers, making them inaccessible or changing their chemical

composition. This means that there is a limiting toxin concentration for this method of detoxification. However, it was reported that L. rhamnosus GG tolerated higher AFB_1 concentration and linearly adsorbed the toxin up to 13.3 µg/ml (EL Nezami, 2003). It is clear that, bacteria that show high binding rate tolerate high toxin concentration. Though 0.5 µg of toxin improved adsorption rate, 1 µg concentration was used through our experiment to test a more practical concentration.

Table (5): Effect of concentration of toxin on its binding by L. casei

Toxin Conc., µg	Toxin removal , %	
	Aflatoxin B_1	Aflatoxin M_1
0. 5	**40. 4**	**31. 9**
1. 0	**33. 40**	**29. 40**
1. 5	**30. 70**	**27. 70**
2. 0	**25. 30**	**21. 90**
5. 0	**16. 50**	**16. 90**

1. Incubation period 2 h at 37°C.
2. Bacterial concentration $1.7x10^{11}$ CFU / ml.

Table (6) presents the effect of reaction pH and bacterial heat and acid killed on toxin binding rate. Binding reaction is favored by acidic pH. There was a significant ($P<0.05$) increase in toxin binding at low PH (2.73) reaching a 40% level compared to 33.9% in the alkaline PH. It was reported that treating bacteria with pH 2, significantly increased binding with L. rhamnosus GG. It was suggested that in the presence of acid some intracellular beside the regular extra-cellular binding occurred (**10**). Heat killed and acid killed microorganisms showed higher rate of toxin binding than viable cells, however, the acid killed was more effective. Both treatments might have made more binding sites to be available for binding in cell wall.

Table (6): Effect of reaction pH and heat on L. casei toxin binding rate:

pH value	Aflatoxin B_1	Aflatoxin M_1
2.73 (acid killed)	**40.01**	**33.30**
4.2	**35.51**	**28.89**
5.5	**34.90**	**28.09**
7.2	**34.10**	**27.70**
7.2(heat killed)	**36.29**	**28.29**
8.1	**33.92**	**26.48**

- **Hcl and NH_4OH were used to adjust the pH values, toxin concentration1µg /1ml PBS.**
- **Adsorption reaction was at 37°C for 2 hrs and the inoculume was$1.7x10^{11}$ CFU / ml.**

It is known that bacteria have great ability to adapt themselves to overcome growth adverse conditions. Therefore, a trial was made to make L. casei to be adapted for AFB_1 and AFM_1. The repeated exposure of the same cells to toxin after their activation in skim milk was tried and results are in Table (7)

Table (7): the effect of repeated exposure of bacteria to toxin on their binding ability:

Number of exposures	Toxin removal, %	
	Aflatoxin B_1	Aflatoxin M_1
1	34.2	27.5
2	43.8	38.7
3	47.1	41.7
4	49.1	43.1
5	50.0001	43.1
6	50	43.1
7	50	43.1

- **L. casei was activated in skim milk after each exposure to reach $1.7x10^{11}$CFU / ml.**
- **Binding incubation period was 2h at 37°C, Toxin concentration 1ug/ ml.**

The ability to bind both toxins increased by repeated exposure. Maximum of 50% binding of aflatoxin B_1 was reached on the fifth exposure, and there was no effect for more exposures. Aflatoxin M_1 binding reached maximum of 43.1% on the fourth exposures with no increase on more exposure. These values of binding were significantly higher than the normal Lb. casei; therefore, this process seems to be important.

To test the applicability of this detoxification method, yoghurt and sweet cultured milk were processed from aflatoxin M_1 contaminated milk using Lb. casei, the toxin binding rates are in Table (8). L. casei removed the toxin during yoghurt processing almost in similar removal values from PBS medium (30.39% in yoghurt compared to 34.1% in PBS for AFB_1). In culture sweet milk, the removal of the toxin was far below the level obtained in PBS(21.2% removal from milk compared to 34.1% in PBS) . In general the removal of toxin from milk particularly skim milk was reported to be in lower percentage then from PBS (**20**). The low values of removal from sweet cultured milk was due two factors first being skim milk and the second was the low temperature of binding reactions. The normal rate of toxin removal from yoghurt was due to the proper temperature during incubation(40°C) and period of binding reaction and the development of acidity during the incubation period (PH~4.5). Therefore, this method of detoxification is valid in dairy products such as ripened cheese and fermented milk. Of course, the treatment would be more feasible by selecting microbial strains that have high toxin binding affinity e.g. Lb. rhamnosus GG. There are number of trials are currently undergoing to improve toxin adsorption from sweet cultured milk.

Table (8): Aflatoxin removal by lactic acid culture from milk during processing yoghurt and sweet cultured milk:

Milk products	Toxin removal,%	
	Aflatoxin B_1	Aflatoxin M_1
Yoghurt [2]	30.39	18.25
Sweet cultured milk [3]	21.2	16.3

1. Toxin concentration 1ug/ ml.
2.Coagulation was at 40°C / 4 hrs.
3.Mixing was at 40°C followed by immediate cooling to 4°C.

The effect of toxin adsorption on bacterial adhesion to the intestine tissue was also studied by exposing both intestine mucus and epithelial cells into Lb. casei and L. casei-toxin complex. Figure (2) shows the binding behavior. It is clear from the photo that Lb. casei – toxin complex whether with aflatoxin B_1 or M_1 did not adhere to both tissues. This is an important advantage for this method of detoxification, that there is no accumulation for the toxin in the intestine thus alleviating its toxicity. To prove this point the bioavailability of the toxin when adsorbed by Lb. casei was determined by examining the effect of feeding rats with free toxin and Lb. casei toxin complex on their livers.

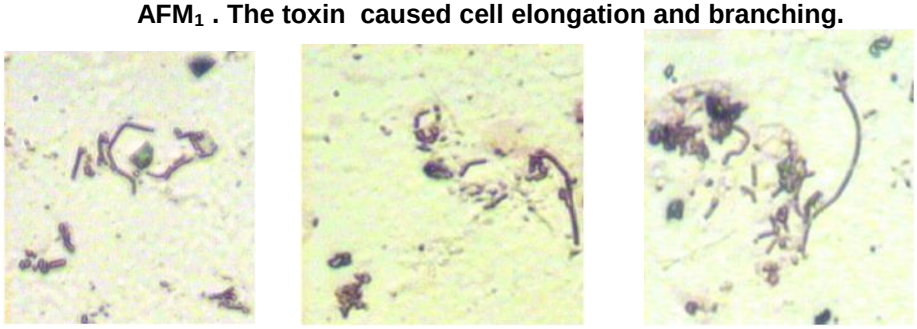
Figure (3) shows normal liver tissue structure of rats. The hepatic lobules are formed of rows of polyhedral hepatocytes containing a nuclei and abundant cytoplasm. There are blood sinusoids in – between the hepatocytes separated from them by endothelial cells. The walls of the sinusoids contain phagocytic irregular cells with multiple processes known as Von Kupffer. The sinusoids run radially, converging at the centre of the hepatic lobule to form the central or centrolobular vein. Figure (4) shows the effect of oral administration of probiotic bacteria on liver tissue structure of rats. The tissues showed normal structure. Figures (5) and (6) present the effect of 3 weeks of oral administration of 21µg aflatoxin B_1 for each rat on their liver tissue. The liver showed macroviscular fatty change and hydropic degeneration (Fig.5). In some rats, congested hepatic sinusoids and mild lymphocyte infiltration was noticed (Fig. 6). Figures (7 and (8) present liver tissues of rats each was fed on total of 21µg AFM_1 orally administered over 3 weeks. The tissues of some rats were almost normal with activated Von Kupffer cells (Fig. 7). In other rats the liver showed focal necrosis associated with lymphocyte infiltration and some vacuolated hepatocytes and pyknotic nuclei (Fig.8).

Figure (9) and (10), point out the effect of oral administration of L. casei – AFB_1 complex prepared from 21µg toxin for three weeks on rats' liver. In most rats, liver hepatocytes appeared more or less normal like the control (Fig. 7). In few rats there was some congestion and dilation of the portal area with microvesicular fatty change (Fig. 10). Figure (11) shows the effect of oral administration of L. casei – AFM_1 complex prepared from21µg toxin for three weeks on rats' liver. The hepatocytes appeared almost normal like the control except few areas of hydropic degeneration. Results pointed out that binding the toxin with LAB alleviated the harmful effect of the toxin on liver tissues.

In conclusion, this is a proper detoxification method for food and dairy products, since LAB could be part of food process and if not the heat or acid killed bacteria bind the toxin as well. These results proved that, the use of LAB for toxin removal prevented probiotic bacteria – toxin complex from the adhesion into intestinal wall preventing its absorption. The complex therefore is excreted out of the body. This was proved by the feeding experiment in which feeding the bacteria – toxin complex showed almost normal liver tissues. It is expected that, the use of other LAB which show higher toxin removal than L. casei would give good results with high aflatoxin

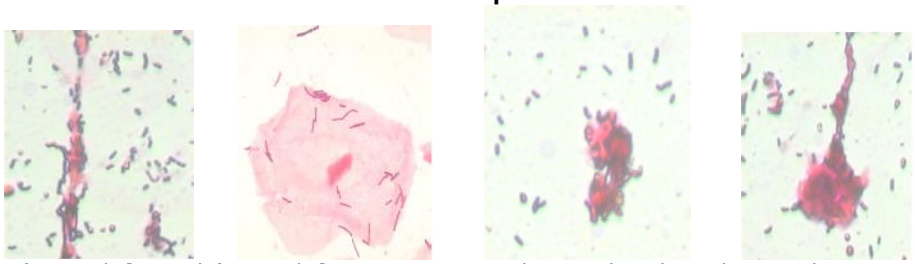
concentrations. Therefore, screening different probiotic bacteria for their rate of toxin removal is recommended to find new high rate toxin removal microorganisms. Moreover, the use of bacteria adaptation method tried in this work would help in improving the rate of adsorption of a particular microorganism needed for a particular process.

Figure (1): L. casei morphology changes when exposed to AFB_1 and AFM_1 . The toxin caused cell elongation and branching.



a- AFB_1 for 48 h b- AFM_1 for 48 h c- AFB1 for 72 h.

Figure (2): L. casei and L. casei –AFB_1 and AFM_1 complexes adhesion into intestinal mucus and epithelial cells.



a- free L. casei adhere to intestinal mucus b- free L. casei adhere to intestinal epithelial cell c- L. casei-AFB complex show no adhere to mucus d- L. casei-AFM complex show no mucus adhere

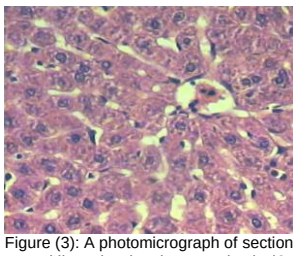


Figure (3): A photomicrograph of section of control liver showing the central vein (CV) lies at the centre of the lobule surrounded by the hepatocytes (HC) with strongly eosinophili c granulated cytoplasm (CY), and distinct nuclei (N).Between the strands of hepatocytes, the hepatic sinusoids are shown (HS) **(H & E X 300)**.

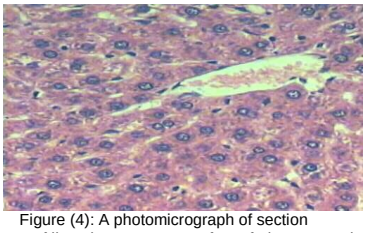


Figure (4): A photomicrograph of section of liver tissue structure of rats fed on L. casei showing normal structure. **(H & E X 300)**.

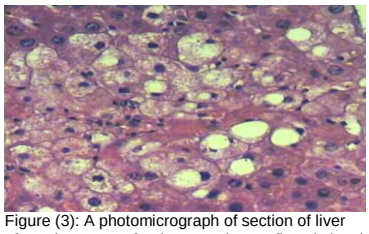


Figure (3): A photomicrograph of section of liver of rat given AFB1 for three weeks at Aflatoxin levels of 21 µg for each treatment showing macroviscular fatty change and hydropic degeneration **(H & E X 300)**.

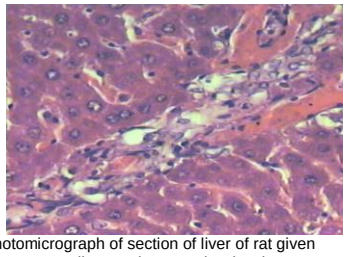


Figure (4): A photomicrograph of section of liver of rat given of total 21 µg AFB_1 orally over three weeks showing congested hepatic sinusoids and mild lymphocyte infiltration**(H & E X 300)**.

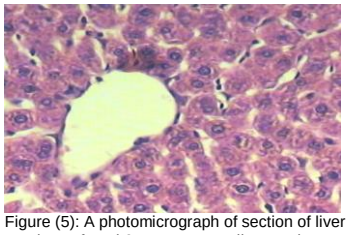


Figure (5): A photomicrograph of section of liver of rat given of total 21 µg AFM_1 orally over three weeks , showing normal structure. Notice the activated Von Kupffer cells **(H & E X 300)**.

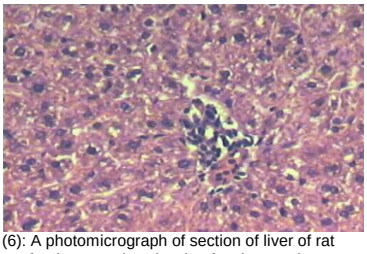


Figure (6): A photomicrograph of section of liver of rat 21µg AFM1 for three weeks, showing focal necrosis associated with lymphocyte infiltration. Some vacuolated hepatocytes and pyknotic nuclei are seen **(H & E X 300)**.

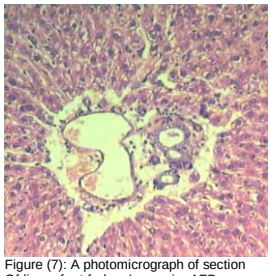


Figure (7): A photomicrograph of section Of liver of rat fed on L. casei – AFB_1 Complex for three weeks, showing mini -mum congested and dilated portal area with microve sicular fatty change (**H & E X 300)**.

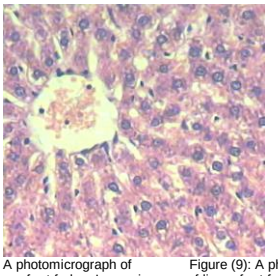


Figure (8): A photomicrograph of section of liver of rat fed on L. casei – AFB_1 complex for three weeks , showing hepatocytes that appear more or less like control (**H & E X 300**)

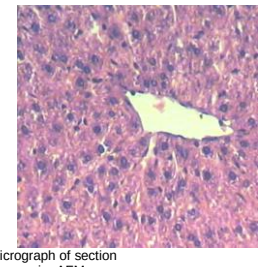


Figure (9): A photomicrograph of section of liver of rat fed on L. casei – AFM_1 complex for three weeks, showing the hepatocytes that appear more or less as control except few areas of hydropic degeneration (**H & E X 300)**.

REFERENCES:

1. Ambrosini V., S. N. Gonzalez, and G. Oliver. 1999. Study of Adhesion of Lb. casei CRL 431-to IIeal intestinal cells of mice. J. food prot. 62(12) 1430 – 1434.

2. Bintvihok A., S. Thiengnen, K. Doi, and S. Kumagai. 2002. Residues of Aflatoxin in the liver muscle and Eggs of domestic fowls. J. Vet. Med. Sci.64 (11):1037 – 1039.

3. Bulter W.H., M. Greenblatt, and W.Liginsky .1969. Carcinogenesis in Rats by Aflatoxin B_1, G_1 and B_2. Cancer res. 29: 2206 – 2211.

4. Clifford J. I. and K. R. Rees. 1976. The Action of Aflatoxin B_1 on Rat Liver. Biochem. J. 102: 65 – 75.

5. Coallier – Ascah J., and E. S. Idziak. 1985. Interaction between streptococcus lactis and aspergillus flavus on production of aflatoxin. App. Environ. Micro. 49(1):163 – 167.

6. D'Souza D. H., and R. E. Brackett. 2001. Aflatoxin B_1 degradation by Flavobacterium aurantiacum in the presence of Reducing conditions and seryl and sulfhydryl Group inhibitions. J. food prot. 64(2): 268 – 271.

7. E. B. Lillehoj, A. Ciegler, and H. H. Hall. 1967. aflatoxin B_1 uptake by Flavobacterium aurantiacum and resulting toxic effects. J. bacteriology, 93(1):464 – 471.

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

9. El–Nezami H., Kankaanpää P., Salminen S., Ahokas J. 1998b: Physicochemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media. J Food Prot 61: 466 – 468.

10. El-Nezami H, Myknen H, Kankaanp P, Salminen S, Ahokas J. 2000. Ability of Lactobacillus and propionibacterium strains to remove aflatoxin B_1 from chicken duodenum. J Food Prot, 63:549-552.

11. El – Nezami H., Polychronaki N., Salminen S., Mykkänen H. (2002a): Binding rather than metabolism may explain the interaction of two food Grade *Lactobacillus* strains with zearalenone and its derivative (ά) alpha zearalenol. Appl. Environ Microbiol. 68:3545 – 3549.

12. Gratz S, Mykknen H, Ouwehand AC, Juvonen R, Salminen S, El-Nezami H: Intestinal mucus alters the ability of probiotic bacteria to bind aflatoxin B_1 In Vitro. Appl. Environ. Microbiol. 2004, 70:6306-6308.

13. Haskard C., Binnion C., Ahokas J. (2000): Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. Chem Biol Interact 128:39 – 49.

14. Haskard C. A., El - Nezami H. S., Kankaanpää P. E., Salminen S., Ahokas J. T. (2001): Surface binding of aflatoxin B1 by lactic acid bacteria. Appl Environ Microbiol 67:3086 – 3091.

15. Kankaanpää P., Tuomola E., El – Nezami H., Ahokas J., Salminen S. J. (2000): Binding of aflatoxin B1 alters the adhesion properties of *Lactobacillus rhamnosus* strain GG in a Caco2 model. J Food Prot 63:412 – 414.

16. Kos B., J. Suskovic, M. Simpraga, J. Frece, and S. Matosic. 2003. Adhesion and aggregation ability of probotrc strain Lactobacillus acidophilus Mg 2. J. Appl. Micro. 94 :981 – 987.

17. Lee, Y.K., H. EL – Nezami, C.A. Haskard, S. Gratz, K.Y. Puong, S.Salminen, and H.Mykkanen.2002. Kinetic of adsorption and desorption of Aflatoxin B, by viable and non viable bacteria. J. food prot. 66 :(3) 426 – 430.

18. Liginsky W., K. Y. Lee, and C. H. Gallaghher. 1970. Interaction of Aflatoxin B_1 and G_1 with tissues of the rat. Cancer res. 30: 2280 – 2283.

19. Lin W., C. Hwang, L. Chen, H. Tsen. 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food micro. 23: 74 – 81.

20. Pierides M., El Nezami H., Peltonen K., Salminen S., Ahokas J. (2000): Ability of dairy strains of lactic acid bacteria to bind aflatoxin M_1 in a food model. J. Food Prot. 63:645 - 650.

21. Peltonen K., H. El – Nezami, J. Ahokas , and S. Salminen. 2001. Aflatoxin B_1 binding by dairy strains of lactic acid bacteria and bifidobacteria. J. dairy sci. 84: 2152 – 2156.

العوامل المؤثرة على إدمصاص بكتيريا حامض اللاكتيك للآفاللاتوكسين وتأثير إدمصاص التوكسين على النسيج الكبدى للفئران

محمد متولى ، السيد عبد الله ، عبد الرازق فرج ، سناء بدران و أحمد نوح
قسم الألبان – كلية الزراعة – جامعة القاهرة

أمكن إستخدام قدرة بكتيريا حامض اللاكتيك على إدمصاص الأفلاتوكسين كوسيلة للتخلص من التوكسين فى معظم منتجات الأغذية والألبان. وتم إستخدام سبعة سلالات من بكتيريا حامض اللاكتيك بالإضافة إلى نوعين من مخاليط البكتيريا التجارية المستخدمة فى تصنيع منتجات الألبان وقياس معدلاتها لإدمصاص نوعاى الأفلاتوكسين B1 وكذلك M1 وقد تم تقدير القابلية الحيوية لإدمصاص التكوسين لتغذية مجموعات من الفئران على مخلوط من معقد البكتيريا مع الأفلاتوكسينات المذكورلاة. وأجريت تجربة لإستخدام ١ مللى من محلول بفر الفوسفات المحتوى على ١ ميكروجرام من التوكسين مخلوطا مع خلايا البكتيريا على درجة ٣٧ مئوى وأس هيدروجينى ٧,٣ لمدة ساعتين وكان أعلى تركيز لإدمصاص التوكسين هو ٣٤,١ ، ٢٧,٧% لكل من نوعى الأفلاتوكسين المستخدمين. وتزداد المعدلات كلما إنخفض تركيز التوكسين. وبإستخدام نصف ميكروجرام أو أعلى من الأفلاتوكسين أدى إلى حدجوث تغيرات مورفولوجية للخلايا البكتيرية. كما التصنيع زبادى ولبن محلى من اللبن الملوث للأفلاتوكسين ووجد إستجابة جيدة لربط نوع التوكسين بواسطة البكتيريا المستخدمة.

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