LACTOBACILLUS PLANTARIUM GE6 REDUCES AFLATOXIN B₁ TOXICITY IN RATS AND CACO-2 CELLS

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Received 6/5/2010 Accepted 11/10/2010

ABSTRACT
Aflatoxin B₁ is potent mycotoxin produced by some Aspergillus species, is a hepatocarcinogen and compromise immunity and interferes with protein synthesis and metabolism in human and animals. In this study we investigated the potential of L. plantarium GE6 to reduce aflatoxin B₁ toxicity in rats and DNA fragmentation in Caco-2 cells line. This work was conducted on 36 male Han-Wister rats which were divided into 3 equal groups each one contain twelve rat. first group received phosphate saline alone and the second group received L. plantarium GE6 (3×10⁷ CFU/0.5ml phosphate saline) at the fourth day of experiment both groups received a single oral dose of aflatoxin B₁ 2ppm/kg in 0.5ml DMSO, third group served as untreated control. There was no significant reduction in body weight gain in rat dosed 2ppm/kg of aflatoxin B₁ alone in comparing with the second group received aflatoxin B₁ and L. plantarium together. Fecal excretion of aflatoxin B₁ increased to 16.2% ±(3.1) within first day after aflatoxin B₁ dose in group receiving aflatoxin B₁ and L. plantarium GE6 compared to the group received aflatoxin B₁ alone 7.4%±(2.3). Liver enzymes were measured in plasma on the sixth day of the experiment, alanine transaminase (ALT), Glutamate oxalic transferase (GPT) and Glutamate pyruvate transferase (GOT) increased significantly (p<0.05) to 124.5, 184.7 and 101 (IU/L) in group exposure to aflatoxin B₁ alone, liver enzymes in group received L. plantarium in addition to aflatoxin B₁ was not differ significantly from control group and it was 70.2, 92.4 and 49.2(IU/L) for ALT, GPT and GOT respectively. DNA fragmentation was assessed in Caco-2 cells exposed to aflatoxin B₁ followed induction of Cytochrome P (CYP3A4), DNA damage occur 28.1% and 52.4% after 24 and 72 hour respectively in cells treated with aflatoxin B₁ only ,a significant reduction (p<0.05) in DNA damage observed after 72 hour only at cells coincubated with 3×10¹⁰ cell/ml of non viable L. plantarium GE6 (36.7% of DNA).

Key words: Aflatoxin B1, Probiotic, Lactobacillus, Carcinogenicity

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تقليل سمية الأفلاتوكسين B1 في الجرذان وخلايا Caco-2 بواسطة Lactobacillus planetarium GE6

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النوع 2/10/2010، الفصول 6/5/2010

الخلاصة

الأفلاتوكسين B1 من السموم الفطرية المنتجة من قبل بعض أنواع الفطر. وهو من Aspergillus spp. من السموم الفطرية المنتجة من قبل بعض أنواع الفطر. العوامل المسرطنة لكبد والمثبطات للجهاز المناعي فضلاً عن تأثيره على عملية تكوين البروتينات وبيضة في L. planetarium GE6. أجريت هذه الدارسة لتقييم قابلية L. planetarium GE6 على تقليل سمية الأفلاتوكسين في الجرذان وعلى تكسر DNA على النواة Caco-2 في الخلايا السرطانية 2. وجدت دراسة DNA على النواة Caco-2 في الخلايا السرطانية 2. وفي النهاية قسمت إلى ثلاثة مجموعات متساوية، المجموعة الأولى جرعت فموياً بحلول phosphate (Han Wister) نقطة. المجموعة الثانية جرعت ببكتريا saline DMSO من المجموعة الأولى والثانية جرعة واحدة من الأفلاتوكسين B1 بتركيز 2ppm/kg في اليوم الرابع من التجربة. المجموعة الثالثة عمت كمجموعة سيطرة. إنخفاض معنوي في وزن الجسم في المجموعة الأولى مقابل مع مجموعة الثانية. فقد أثر طرح الأفلاتوكسين خارج الجسم عن طريق الفضلات بشكل معنوي (P<0.05) في اليوم الأول بعد جرعة الأفلاتوكسين B1 في المجموعة الثانية مقابل مع المجموعة الأولى المعالمة بالأفلاتوكسين لوحدة (2.3) ± 7.5%. قيم تراكيز إنزيمات الكبد في اليوم السادس من التجربة 91FGPT, GOT and ALT 06112 من تراكيز إنزيمات الكبد في اليوم السادس من التجربة 91FGPT, GOT and ALT. ولم يكن التغير معنوي على مستوى القيم من ALT الكبد في المجموعة الثانية مقابل مع مجموعة السيطرة وكانت 49.2, 70.2, 29.4 لكل من ALT على التوالي. Caco-2 في الخلايا السرطانية 2. قيم التكسر الحاصل في الـ Caco-2 في الخلايا السرطانية 2. وقد وجد أن نسبة تكسر الـ DNA هي 28.1% و 52.4% بعد 24 و 72 ساعة على التوالي في CYP3A4 والخلايا المعالمة بالأفلاتوكسين فقط، بينما لوحظ إنخفاض بشكل معنوي في نسبة تكسر DNA قبل معالمة الأفلاتوكسين. بقيت نسبة الإنخفاض في تكسر DNA في الخلايا التي تم تغذيةها بـ DNA بعد تفاعلها مع الأفلاتوكسين 3 ×10^7 CFU/ml من الخلايا الفرخية بنسبة 36.7%.
INTRODUCTION

Aflatoxin B₁ (AFB₁) is a potent mycotoxin produced by some species of Aspergillus. This toxin is one of the most hazardous mycotoxins, is extremely toxic, mutagenic and carcinogenic (1,2). It poses a severe threat to both livestock productivity and human health and thus, brings huge worldwide economic losses each year (3). Contamination of AFB₁ in feed cause aflatoxicosis in poultry production characterized by anorexia with lowered growth rate, poor feed utilization, decreased weight gain, decreased egg weight and production, increased susceptibility to environmental and microbial stresses, and increased mortality (4). Aflatoxin B₁ can cause important gross and microscopic changes in the liver, such as hepatomegaly, paleness, hydropic degeneration, fatty changes, bile duct hyperplasia and periportal fibrosis (5,6) impairment of the humoral and cellular immune responses and increase susceptibility to some environmental and infectious agents (7). Aflatoxin B₁ form a covalent adducts with macromolecules such as proteins and DNA (8). This reactive is capable of causing damage to cells in the liver and at the intestinal interface. Various physical and chemical methods have been developed and tested for controlling AFB₁. However, disadvantages of these methods, such as nutritional loss, sensory quality reduction and high cost of equipments, have limited their practical applications (9,10). It is expected that progress in the control of mycotoxins contamination will depend on the introduction of technologies for specific, efficient, and environmental detoxification. The utilization of microorganisms or their enzymatic products to detoxify mycotoxins in contaminated food and feed can be choice of such technology (11,12). Lactobacillus species were well known for their systematic beneficial effects on human and animals and that make it more suitable for feed and food additives (13). Previous studies had shown that many species of Lactobacillus strains, efficiently remove AFB₁ from solution in vitro (14,15). Theoretical calculations by Oatley et al. (16) demonstrate that AFB₁ removal does not arise solely from trapping of the toxin in the bacterial pellet during centrifugation. Metabolic conversion and covalent binding of AFB₁ by the bacteria have been excluded as a mechanism of removal, and noncovalent binding of AFB₁ to the bacteria has been proposed. These strains reduce tissue uptake of AFB₁ from the duodenum of chicks (17) and may permit detoxification of the human diet through reducing aflatoxin absorption in the gastrointestinal tract. It is important that nonviable bacteria also have high binding ability, as survival of viable bacteria is reduced upon passing through the stomach at low pH. Similar mutagen-binding abilities have been reported for viable and nonviable (heat-treated) bacteria (18,19). Nonviable (heat-treated) strains bind AFB₁ as effectively as viable bacteria. The aim of this study is biological evaluation of the efficiency of Lactobacillus planetarium GE6 isolate on reducing the toxic effect of aflatoxin B₁ in rats.
MATERIAL AND METHODS

Bacterial Strains

*Lactobacillus planetarium GE6* was obtained from Institute of genetic engineering and biotechnology/ Baghdad University, as lyophilized powder and stored at -80°C. Bacteria were suspended in phosphate-buffered saline, boiled for 1 h in a water bath, and centrifuged (5,000 rpm for 10 min, 4°C). After being washed once with culture medium DMEM (Dulbecco's modified Eagle's medium) containing antibiotics/antimycotics (0.2% penicillin, streptomycin, amphotericin B; Oxoid, United Kingdom), bacteria were centrifuged again and then suspended in DMEM containing antibiotics, resulting in final concentrations of 3 x 10^10 cell/ml.

Cell line and culture conditions

The human colon cancer cell line caco-2 (ATCC, HTB-37) was cultivated in DMEM, then 20% fetal bovine serum was added and 0.2% antibiotic (20IU/ml pencilline, 20 mg/ml Streptomycine, 0.05 mg/ml streptomycine B). Culture was kept at 37°C with 5% CO2 for 21 days to allow cells differentiation.

Induction with 1α,25 dihydroxy vitamin D3

The cell line has low expression of CYP 3A4 enzyme that generates the aflatoxin epoxide. This enzyme was induced by pre-incubation the tissue with 250 mM 1, 25(OH)2D3 in 0.05%ethanol for 72 h (20).

AFB1 induced DNA damage

Differentiated Caco-2 cells were cultured in the presence of 1,25(OH)2D3 for 5 days prior to AFB1 exposure. Following 72 h of incubation with AFB1 alone (150 µM) or with *L. planetarium GE6* (1 x 10^10 or 5 x 10^10 CFU/ml) and AFB1 (150 µM), the cells were harvested using trypsin-EDTA and viability was assessed with trypan blue staining. Only cell cultures with viabilities of >90% were used for further assays. DNA damage was assessed using a DNA fragmentation assay. The DNA fragmentation assay includes lysis of the cells, extraction of DNA with phenol-chloroform, and gel electrophoresis as described in detail (21).

Animals and Expermintal design

Animal experiments were carried out in the animal facilities within the Pathology department/Medicine college/Malaya university in Malaysia. Thirty six rats (Five weeks old) Han-wister rats were used, these rats divided into 3 groups.

**Group 1**: twelve rats received PB only for six days and provided by oral gave single AFB1 dose in the third day (2µg/Kg body weight in 0.5ml DMSO).

**Group 2**: twelve rats received *L. plantarium GE6* (3x10^7 CFU/0.5 ml PB), for six days and provided by oral gaves single AFB1 dose in the third day(2µg/Kg body weight in 0.5ml DMSO)

**Group 3**: twelve untreated rats (control group).

Data Collection

**Body weight**: body weight for each group was recorded at the beginning of the study (prior to Bacterial treatment).on the day of AFB1 dosing, and at the end of the study.
Liver Enzymes: at the sixth day rats were anesthetized by CO2 inhalation and blood samples were taken by cardiac puncture into heparinized 5 ml blood collection tubes. After centrifugation (5000rpm/10min/4°C), the plasma samples were stored at -20°C. ALT, GOT and GPT activity used as a marker for liver injury caused by AFB1, a commercial kit were used to measure enzyme activities (Boehringer, Mannhei, Germany).

3.2.9 Quantification of fecal AFB1 levels
All fecal samples were collected daily, AFB1 extracted from feces by mixing with acetonitril for 30 minutes, centrifuge this mixture at 7000 rpm/10 min/10°C, after that remove the precipitate and the supernatant dried by centrifugation under vaccum, and kept the dry samples in refrigerator at -20°C. Quantification of AFB1 was performed by added 0.2 ml from acetonitril to the dry samples and mixed well in vortex for one minutes, then analysis by analytical HPLC.(22).

Analytical HPLC conditions
Shimadzu (UFLC-PDA-IT-TOF) system consist of dual pump solvent delivery system. Programmable fluorescence detector and water XBidge 2.2×50 mm column. The sample injection volume of auto injector was set to 100 ml, the solvent system H2O (formic acid 0.15) methanol/acetonitril 6:4 as a mobile phase, the flow rate 0.5 ml/min.

Statistical analysis
The results obtained were analyzed for significant differences using Duncan multiple range test and general linear model (GLM) procedure of SAS software (SAS Institute, 1992). all treatment of significance are based on the probability level of 0.05.

RESULTS AND DISCUSSION

Body weight
In this study the body weight of animals in the beginning of the study was similar in all groups (152.0±22.1 for untreated controls, 153.0±8.0 for the group AFB1 only, and 154.1±7.9 for the group AFB1+GE6). During the duration of the study the untreated control animals gained 7.0±2.0 g/day. Prior to AFB1 dosage the daily body weight gain of the treatments groups were similar (6.3±1.1, and 6.1±1.2 g/day, for AFB1 alone and AFB1+GE6, respectively) and there was no clear differences from the body weight gain in untreated controls.
Figure (1): Effect of *L.* *plantarium GE6* on daily body weight gain after a single dose of AFB$_1$ (2µg/kg of body weight) in rats.

Absences of significant differences between all treatments suggesting that may be due to short time of aflatoxin exposure (3 days) since enhance intestinal damage exert by AFB$_1$ and decrease boday weight its very varied among the same animal species and may be needed to more exposure time to sign significant differences (23).

**Liver Enzymes**

The activity of alanine transaminase (ALT) in plasma, an indictor of liver injury, was highly variable. ALT activity was similar in the control and AFB$_1$ plus *L.plantarum GE6* group (56.2±18.7, and 70.2±24.2 U/l, respectively, p=0.317), while a trend towards increased ALT activity was seen in the group receiving AFB$_1$ alone (124.5±34.2 U/l, p=0.053) as compared to controls.

Concerning the GPT and GOT, value significantly increased, passing from 69.3 ± (18.7) to 184.7 ± (22.9) and from 41.5 ± (3.8) to 101.3 ± (11.6) respectively for groups receiving AFB$_1$ alone in comparing with control (Table 1), while there was no significant differences between control and group receiving AFB$_1$ in addition to *L.plantarum GE6*, that means clearly *L.plantarum GE6* reduced the toxic effect of AFB$_1$ which cause increasing of liver enzymes value results of liver injured.
Table(1): Level of liver enzymes in rats exposure to 2 mg/kg aflatoxin of body weight.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Control</th>
<th>AFB₁</th>
<th>AFB₁/GE6</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT(IU/L)</td>
<td>56.2⁺</td>
<td>124.5⁺</td>
<td>70.2⁺</td>
</tr>
<tr>
<td>GPT(IU/L)</td>
<td>69.3⁺</td>
<td>184.7⁺</td>
<td>92.4⁺</td>
</tr>
<tr>
<td>GOT(IU/L)</td>
<td>41.5⁺</td>
<td>101.3⁺</td>
<td>49.2⁺</td>
</tr>
</tbody>
</table>

a,b different letters between treatments refer to significant differences (P<0.05).

These enzymes are found out in several tissues: liver, heart, kidney, muscles spleen and intestine; therefore, it is not specific to a particular organ, but its activity is more important in the liver, the heart and the muscles (24). Those two enzymes are often carried out in a coupled way. When the GOT and the GPT increase simultaneously, a liver attack is suspected (25,26).

The increase in the GOT and GPT plasma rate following the aflatoxin exposure could cause a malfunction of those organs especially at the level of the liver. In fact, it is probable that the increase in the GOT and GPT activity is constant among men suffering from a severe hepatitis (22). In addition, Balint et al. (27) emphasize that the increase in the GOT and GPT rate is a sign of tissue necrosis, more particularly a hepatic one. Jelinska et al. (28) also indicated that, among cattle and sheep, the GOT plasma values undergo an important increase during acute liver ailments.

Fecal excretion of AFB₁

Bacterial AFB₁ binding was tested in vivo by measuring AFB₁ levels in feces of rats for 3 days following a single oral AFB₁ dose. Fecal excretion of AFB₁ was increased by more than one fold to 16.2% of the initial dose that given to the rats within the first day after the AFB₁ dose in the group receiving AFB₁ and GG as compared to the group received AFB₁ alone 7.4%, whereas no difference was observed on days 2 and 3 after AFB₁ dosing. Fecal excretion was 4.2% and 4.5% for group receiving AFB₁ alone on days 2 and 3 respectively and it was 6.3% and 4.8% for group receiving AFB₁ and L.plantarum GE6 on days 2 and 3 respectively Figure (2).
Figure(2): Effect of *Lactobacillus plantarium* GE 6 on daily fecal AFB<sub>1</sub> excretion over three days following a single dose of AFB<sub>1</sub>.

The percentage of AFB<sub>1</sub> bound by probiotics in feces can be calculated by comparing AFB<sub>1</sub> levels in rats dosed with or without probiotic administration. This shows that additional AFB<sub>1</sub>/24 hour feces are excreted in feces due to probiotic binding. Under *in vivo* conditions, one would expect probiotic bacteria to bind AFB<sub>1</sub> as soon as they interact with each other inside the intestinal tract. Thereafter bacteria should travel through the intestinal tube and be excreted eventually into feces, taking bound AFB<sub>1</sub> with them. Consequently, fecal levels of AFB<sub>1</sub> should allow us to estimate AFB<sub>1</sub> binding occurring *in vivo*, even though a percentage of binding calculated from results obtained from rat fecal samples lies well below results *in vitro* or *ex vivo*.

Assessment of AFB<sub>1</sub> induced DNA damage

To exert toxic effects, aflatoxin B1 has to be activated to the highly reactive AFB<sub>1</sub> 8, 9 epoxide (1,29), which then adducts to proteins and DNA. Caco2 cells lack the expression of CYP 3A, the key enzyme to facilitate this activation, and 1,25(OH)2D3 has to be used to induce the expression of this enzyme.

As marker of AFB<sub>1</sub> induced DNA damage in the cells, DNA fragmentation was assessed in Caco2 cells exposed to AFB<sub>1</sub> following induction of CYP 3A4 induction. In this study the Comet assay was used to quantify the extent of DNA damage. No DNA damage was seen in the comet assay when Caco2 cells were incubated either GG, 1, 25 (OH) 2D3 or AFB<sub>1</sub> alone. DNA damage occurred after incubating 1,25(OH)2D3 induced Caco2 with AFB<sub>1</sub> (2ppm) 28.1%±9.3 and 52.4%±14.0% tail DNA for 24 and 72 hours.
A significant (p<0.001) reduction in DNA damage was observed after 72 hours only at the cells concentration (3x10^{10} CFU GG/ml) (36.7±11.8% tail DNA) Figure(3).

Figure(3): Effect of heat treated *Lactobacillus plantarium* GE6, and 3x10^{10} CFU/ml on DNA damage in differentiated Caco2 cells (following induction with 1, 25(OH) 2D3 for 5 days).

These findings suggest that induction of the AFB_{1} bioactivation was not sufficient to reach CYP 3A levels observed in vivo. These results demonstrate that *L.plantarum* GE6 was able to reduce AFB_{1}, formation of aflatoxicol and DNA damage. However, the use of this cell culture model to study effect of probiotic aflatoxin binding has major limitations because AFB_{1} has to be applied in a high concentration and for a long time (up to 72 hours) for formation of AFB_{1} metabolites and damage of DNA in Caco2 cells to occur.

Essigmann *et al.* (30) stated that hepatic DNA from the liver of rats injected with AFB_{1}, nearly 80% of the adduct present are AFB_{1}-formamidopyrimidine constitutes about 7%. The alkylation of DNA with AFB_{1} may also lead to loose of a DNA base, resulting in apurinic site, and at least twice as many apurinic sites detected as AFB_{1}-N7-Gua in synthetic oligomers treated with AFB_{1} Chu and Saffhill, (31). In addition to alkylating DNA, AFB_{1} can induce reactive oxygen species formation, Guindon *et al.* (32) mentioned that DNA damage might be caused by the AFB_{1}-mediated stimulation of reactive oxygen species formation which leads to the oxidation of DNA base.
REFERENCES


