



HAL
open science

In vivo assessment of the potential protective effect of *Lactobacillus casei* Shirota against aflatoxin B1

Adrián Hernandez-Mendoza, Doralinda Guzman-De-Peña, Aarón Fernando
González-Córdova, Belinda Vallejo-Córdova, Hugo Sergio Garcia

► **To cite this version:**

Adrián Hernandez-Mendoza, Doralinda Guzman-De-Peña, Aarón Fernando González-Córdova, Belinda Vallejo-Córdova, Hugo Sergio Garcia. In vivo assessment of the potential protective effect of *Lactobacillus casei* Shirota against aflatoxin B1. Dairy Science & Technology, 2010, 90 (6), 10.1051/dst/2010030 . hal-00895770

HAL Id: hal-00895770

<https://hal.science/hal-00895770>

Submitted on 11 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

In vivo assessment of the potential protective effect of *Lactobacillus casei* Shirota against aflatoxin B₁

Adrián HERNANDEZ-MENDOZA^{1,2}, Doralinda GUZMAN-DE-PEÑA³,
Aarón Fernando GONZÁLEZ-CÓRDOVA², Belinda VALLEJO-CÓRDOBA²,
Hugo Sergio GARCÍA^{1*}

¹ UNIDA-Instituto Tecnológico de Veracruz, M.A. de Quevedo # 2779, Col. Formando Hogar, Veracruz, Ver. 91897, México

² Centro de Investigación en Alimentación y Desarrollo, A.C. – Departamento de Tecnología de Alimentos de Origen Animal, Carretera a la Victoria km 0.6, Hermosillo, Son. 83000, México

³ Departamento de Biotecnología y Bioquímica, CINVESTAV-Campus Guanajuato, Apdo, Irapuato, Guanajuato, México

Received 22 March 2010 – Revised 16 April 2010 – Accepted 30 April 2010

Published online 2 August 2010

Abstract – Interest in the bacterial protective effect against mycotoxins has greatly increased. This effect is mostly strain-dependent and the mechanisms involved are still not well understood. The objectives of this work were (1) to assess the ability of *Lactobacillus casei* Shirota to bind aflatoxin B₁ (AFB₁) by fluorescent monoclonal antibody staining, (2) to evaluate the AFB₁-bacteria interaction by atomic force microscopy, and (3) to determine its effect on intestinal absorption through detection of AFB₁-Lys adducts used as biological markers to a 3-week aflatoxin exposure in a murine model. The micrographs obtained in this work showed for the first time a clear visual image of the ability of *Lactobacillus casei* Shirota to bind AFB₁ into the bacterial cell envelope. The images also revealed that aflatoxin binding produces structural changes that modify the bacterial cell surface. AFB₁-Lys adducts quantified from blood samples were found to be present at significantly lower levels in animals receiving AFB₁ plus bacteria than in those receiving only AFB₁. This suggests that the presence of *Lactobacillus casei* Shirota can decrease aflatoxin absorption at the intestinal level even after a long period of toxin exposure, which consequently circumvents its toxic effects.

murine model / immunofluorescence / aflatoxin B₁ / cell surface / *Lactobacillus casei* Shirota

摘要 – 体内测定 *Lactobacillus casei* Shirota 菌株对黄曲霉毒素 B₁ 潜在的抑制作用。关于细菌抑制真菌毒素的作用已经成为研究热点。这种作用多数是由菌株的特性而决定，而且作用的机制尚不完全清楚。本文 (1) 采用荧光单克隆抗体染色法测定了 *Lactobacillus casei* Shirota 菌株对黄曲霉毒素 B₁ (AFB₁) 的粘附作用; (2) 采用原子力显微镜评价 AFB₁-细菌之间的相互作用; (3) 将实验鼠暴露在黄曲霉毒素环境中 3 周以建立小鼠中毒模型，根据测定 AFB₁-Lys 络合物来评价该菌株对肠道吸收作用的影响。从显微照片上可以清楚地看到 *Lactobacillus casei* Shirota 将 AFB₁ 粘附到细胞膜内。这就说明由于黄曲霉毒素的粘附作用，改变了细菌细胞表面，因而使得细菌的结构改变。根据对血浆中 AFB₁-Lys 络合物的定量测定，黄曲霉

*Corresponding author (通讯作者): hsgarcia@itver.edu.mx

毒素+细菌实验组与只有黄曲霉毒素实验组比较,前者血浆中 AFB₁-Lys 络合物浓度明显低于后者。本实验证明了 *Lactobacillus casei* Shirota 可以显著地降低黄曲霉毒素在肠道中的吸收作用,即使长时间暴露在黄曲霉毒素中也可以避免毒素的危害。

小鼠模型 / 免疫荧光法 / 黄曲霉毒素 B₁ / 细胞表面 / *Lactobacillus casei* Shirota

Résumé – Évaluation in vivo de l'effet protecteur potentiel de la souche Shirota de *Lactobacillus casei* contre l'aflatoxine B₁. L'intérêt porté à l'effet protecteur des bactéries contre les mycotoxines a considérablement augmenté. Cet effet est majoritairement souche-dépendant et les mécanismes impliqués ne sont pas encore bien expliqués. Les objectifs de cette étude étaient (1) d'estimer la capacité de la souche Shirota de *Lactobacillus casei* de lier l'aflatoxine B₁ (AFB₁) par marquage à un anticorps monoclonal fluorescent, (2) d'évaluer l'interaction bactérie-AFB₁ par microscopie à force atomique, (3) de déterminer ses effets sur l'absorption intestinale par la détection des composés AFB₁-Lys utilisés comme marqueurs biologiques après exposition de 3 semaines en modèle de souris. Les micrographies obtenues dans cette étude montraient pour la première fois une représentation visuelle claire de la capacité de *Lactobacillus casei* Shirota à lier AFB₁ au sein de l'enveloppe de la cellule bactérienne. Les images montraient également que la liaison de l'aflatoxine produit des changements structuraux qui modifient la surface de la cellule bactérienne. Les composés AFB₁-Lys quantifiés à partir des échantillons sanguins étaient présents à des niveaux significativement plus faibles chez les animaux recevant AFB₁ plus bactérie par rapport à ceux recevant seulement AFB₁. Ceci suggère que la présence de *Lactobacillus casei* Shirota peut diminuer l'absorption de l'aflatoxine au niveau intestinal même après une longue période d'exposition à la toxine, ce qui limite considérablement ses effets toxiques.

modèle murin / immunofluorescence / aflatoxine B₁ / surface cellulaire / *Lactobacillus casei* Shirota

1. INTRODUCTION

Aflatoxins are common contaminants of foods, particularly in the staple diets of many developing countries. These mycotoxins are produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* during production, harvest, storage, and food processing [38]. The International Agency for Research on Cancer has classified aflatoxins as a group 1 human carcinogen, being aflatoxin B₁ (AFB₁) the most potent and potentially lethal metabolite [24]. Acute exposure to aflatoxins can result in aflatoxicosis, which manifests as severe, acute hepatotoxicity with a case fatality rate of ~ 25%, while chronic aflatoxin exposure is associated with hepatocellular carcinoma. Additional effects include immunologic suppression, impaired growth, and nutritional interference. More than five billion people in developing countries worldwide are at risk of chronic exposure to aflatoxins through contaminated foods [33]. Different strategies

for reducing toxic effects of aflatoxins by consumption of contaminated food have been developed, which include enterosorption and chemoprotection methods. Enterosorption is the use of clay, such as sodium calcium aluminosilicate and zeolitic minerals. Clay acts as an aflatoxin enterosorbent that tightly and selectively binds these poisons in the gastrointestinal (GI) tract, thereby decreasing their bioavailability and associated toxicities. However, since clay and zeolitic minerals comprise a broad family of functionally diverse chemicals, there may be significant hidden risks associated with their indiscriminate inclusion in the diet [28]. On the other hand, chemoprotection involves the use of chemical agents (e.g. phenolic antioxidants, chlorophyllin) or dietary components (e.g. broccoli sprouts sulforaphane, green tea polyphenols) either to protect against the initiation of carcinogenesis or to retard the progression of neoplastic disease once it has begun. This strategy, however, is expensive and is therefore difficult

to implement in poor communities [11, 14]. Recently, the interest in microbial detoxification methods of AFB₁ has greatly increased. Several fungal species have been found to be able to transform AFB₁ into less toxic metabolites. Reduction of AFB₁ by bacteria has also been reported; most of the published studies are focused on lactic acid bacteria (LAB), such as strains from the *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, and *Lactococcus* genera [12]. In vitro studies have reported that bacterial concentration influences the AFB₁ removal. Approximately a minimum of $2\text{--}5 \times 10^9$ CFU·mL⁻¹ is required for significant AFB₁ removal (13–50%), while a concentration of 2×10^{10} CFU·mL⁻¹ is capable of reducing the AFB₁ level to < 0.1% and 13% [2, 5]. The effect of various AFB₁ concentrations on AFB₁ removal has also been tested. The amount of AFB₁ removed increased with increasing concentration of AFB₁ but the percentage removed was not significantly different [5]. On the other hand, a comparison between the removal ability of viable and nonviable bacteria has been previously reported [6, 35] with nonviable bacteria providing the most effective removal, which suggests that AFB₁ reduction seems to be mainly by cell binding rather than metabolism or degradation. However, the binding mechanisms are yet not well understood [16, 21]. Moreover, related trials have revealed that the binding ability is mostly strain-dependent [15, 16]. In this respect, *Lactobacillus casei* Shirota has previously exhibited high affinity for binding AFB₁ in aqueous solution [13, 15, 16]. Ex vivo and in vivo assays have evidenced that LAB may be a safe means to reduce absorption and increase excretion of AFB₁ from the body when administered in a single dose, respectively [7, 9]. Despite this, further studies are needed to overcome the limitation of ex vivo methods to simulate intestinal conditions, and to fully understand the potential of LAB to reduce AFB₁ absorption at intestinal level under chronic toxin exposure. We have

chosen to focus on *Lactobacillus casei* Shirota due to its recognized probiotic status and its proved capacity to bind aflatoxin in vitro. The aims of this work were to assess the ability of *Lactobacillus casei* Shirota to bind AFB₁ by fluorescent monoclonal antibody staining, to evaluate the AFB₁-bacteria interaction by atomic force microscopy (AFM), and to determine its effect on intestinal absorption through detection of AFB₁-Lys adducts as biological markers to a 3-week aflatoxin exposure using a murine model.

2. MATERIALS AND METHODS

2.1. Bacterial strain and culture propagation

The *Lactobacillus casei* Shirota used in this work was isolated from a fermented dairy product. Briefly, MRS broth (pH 6.2) was inoculated with 0.1 mL sample of product and incubated at 37 °C for 14 h. After incubation, a 0.5 mL sample of culture was placed into MRS broth added with bile salts (Oxgall, 1.5 g·L⁻¹; Sigma Chemical Co., St. Louis, USA) [27]. Then, culture was incubated at 37 °C for 12 h. The isolated bacterium was examined by comparing both their bacterial morphology and biochemical profile with descriptions contained in Bergey's Manual of Systematic Bacteriology [18]. The bacteria were maintained by routine subculture at 4 °C in slant tubes with MRS agar. Prior to initiation experiments, the bacterial strain was subcultured twice in MRS broth. Both subculture steps involved 0.1% inocula with incubation at 37 °C for 12 and 8 h, respectively. Then, an aliquot (1%) was transferred into 500 mL of fresh MRS broth (pH 6.0). Culture was incubated without shaking during 20 h at 37 °C. Cells were harvested by centrifugation (3214× g, 10 min, 10 °C) and washed twice with phosphate-buffered saline solution (PBS, pH 7.2) and once with

sterile Milli-Q water. After that, the bacterial pellet was resuspended in 20 mL of sterile PBS. Then, they were used as working cells for further experiments. Bacterial population was determined using the pour-plate method [37], and results were expressed as colony-forming units per milliliter (CFU·mL⁻¹).

2.2. In vitro aflatoxin binding assay

In order to assess the ability of *Lactobacillus casei* Shirota to bind AFB₁ by fluorescence microscopy (FM), four experimental procedures using either FITC-labeled monoclonal antibody against AFB₁ (Ab-FITC (HyTest Ltd., Turku, Finland)), Bacteria, and/or AFB₁ were proposed as follows: (1) Bacteria, (2) Bacteria + Ab-FITC, (3) Bacteria + AFB₁, and (4) Bacteria + AFB₁ + Ab-FITC. Prior to labeling working cells, AFB₁ (Sigma Chemical Co., St. Louis, USA) working solution (4.0 µg·mL⁻¹) was prepared in PBS solution as described in previously published studies [15, 16] and stored in the dark at 4 °C until used. Each experimental procedure was carried out using 0.5–1 × 10⁹ CFU·mL⁻¹ and was performed as described below.

2.2.1. Experimental procedure with working cells

The four experimental procedures proposed were conducted in two stages. Stage A: The microorganisms recovered by centrifugation (3214× g, 10 min, 10 °C) from 1 mL of bacteria suspended in PBS were resuspended in 1.5 mL of the working solution of AFB₁ in procedures 3 and 4, while bacteria in procedures 1 and 2 were resuspended in 1.5 mL of PBS. The mixtures were allowed to react 4 h at 37 °C. Subsequently, cells were recovered by centrifugation and washed twice with PBS in order to eliminate the toxin that was not bound by the bacteria in the respective procedure. Stage B: The pellets were suspended in 300 µL of sterile distilled water,

then 10 µL of Ab-FITC (0.1275 mg·mL⁻¹) were added in procedures 2 and 4; whereas in procedures 1 and 3, 10 µL of sterile distilled water were added. Samples were gently stirred and incubated for 2 h at 37 °C in the dark. After incubation, bacteria were harvested by moderate speed centrifugation (1157× g, 10 min, 4–10 °C) and washed twice with PBS to discard the unreacted antibodies. Collected cells were suspended with 200 µL of antifade solution (50 mg *p*-phenylenediamine 5 mL⁻¹ PBS and 45 mL of glycerol (90%)) and evaluated by FM.

2.2.1.1. Evaluation of working cells by FM

In order to determine the binding of AFB₁ to cell surface of *Lactobacillus casei* Shirota immunofluorescence staining was evaluated by FM. In brief, a 10 µL aliquot of bacteria in antifade solution was placed on a microscope slide under a coverslip. The slides were then examined in an Zeiss Axioskope 2 plus (Jena, Germany) deconvolution fluorescence microscope equipped with a motorized stage, a digital controller of temperature (Zeiss, 37-2) set to 25 °C, a mercury arc lamp (488 nm, Fluo Arc), and a digital camera (Zeiss, AxioCam MRC). Pictures were taken using the Zeiss Axionvision 4.5.0.0 software and the 100 X immersion objective (NA 1.3, Plan-NeoFluar).

2.2.1.2. AFB₁-Bacteria interaction assay by AFM

To evaluate the AFB₁-*Lactobacillus casei* Shirota interaction, both bacteria with and without AFB₁ treatment were analyzed with AFM as follows: a 10 µL aliquot taken from bacteria suspended in 300 µL of sterile distilled water (one step before antibody addition in experimental procedure Stage B) was diluted in 50 µL of sterile distilled water, then, 5 µL of sample were applied on a cover glass slide

previously treated with 20 μL of Concanavalin A (Sigma, $1 \text{ mg}\cdot\text{mL}^{-1}$) as described by Pringle et al. [29]. Bacteria adherent to the coverslip were allowed to dry at room temperature for about 5 min, then the excess of bacteria was removed by washing with 100 μL of sterile distilled water. The coverslip was attached to a metallic disk with double-faced tape and analyzed by tapping mode using an atomic force microscope (Bioscope, Digital Instruments; Santa Barbara, USA) equipped with an inverted light microscope (Zeiss). Experiments were conducted using a silicon tip (Nano-Metrology Probes) with a resonant frequency of 75 kHz in air. The angle and speed of scanning were 90° and 0.150 Hz (256 pixels by line scan), respectively. Every scan resulted in a topography image, which was acquired with the NanoScope (R) IIIa version 5.31R1 software (Digital Instruments Inc., Tonawanda, USA). The imaging session began by using the light microscope and moving the x - y stage in the search for bacteria; the AFM cantilever was then moved toward the surface in the proximity of the chosen bacterium. A large scan (15 μm by 15 μm) was performed in order to assess the exact position and nature of the bacterium, with further smaller scans performed to zoom in on any interesting features. Bacteria were scanned in both directions several times before an image was captured.

2.3. Effect of *Lactobacillus casei* Shirota on AFB₁ absorption at intestinal level under toxin exposure

For in vivo assay, 15 male Wistar rats (200 \pm 20 g) were obtained from Harlan Teklad Inc. (Mexico City) and were housed individually in stainless cages under controlled temperature ($21 \pm 1^\circ\text{C}$), humidity (40–50%), and light (12 h light-dark cycle) conditions with a solid conventional diet (Rodent diet 2018S, Harlan Teklad Inc.,

Mexico City) and water ad libitum. After a 5-day adaptation period, rats were randomly divided into three groups, each group included five animals. The first group of rats served as a control (untreated); the second group (bacteria plus AFB₁) was first given bacterial suspension (5.3×10^8 CFU *Lactobacillus casei* Shirota mL^{-1} PBS) daily during a week, followed by administration of 1.03 mg AFB₁ ($4.0 \text{ mg}\cdot\text{kg}^{-1}$ of body weight) deliberately supplied in seven subdoses over 21 days (experimental period), via oral gavage. Additionally, the probiotic suspension was also supplied at specific intervals of the experiment in order to maintain the bacteria implanted. The third group (treatment without bacteria) received AFB₁ alone in the concentration and subdoses as described above. At specified intervals of the experimental period, blood samples were collected from all groups of rats using the tail tip cut technique described by the Institutional Animal Care and Use Committee [17] to quantify AFB₁ in serum. The currently favored method of measuring aflatoxin exposure consists of the analysis of body fluids for the presence of aflatoxin derivatives. The aflatoxin-albumin adduct is measured in peripheral blood and has a half-life in the body of 30–60 days. Therefore, increased serum AFB₁-Lys adducts are a reliable indicator of chronic exposure [6]. The extraction and purification of adducts from collected samples was performed according to Chapot and Wild [3]. Adduct levels were quantified by competitive enzyme-linked immunosorbent assay (ELISA) using an appropriate test kit (RidaScreen[®] Fast Aflatoxin), with a limit of detection $< 1.70 \mu\text{g}\cdot\text{kg}^{-1}$. The procedure was carried out as described in the manual supplied by the manufacturer (R-Biopharm AG, Darmstadt, Germany), and results were expressed as μg AFB₁-Lys $\cdot\text{mg}^{-1}$ albumin.

2.4. Statistical analysis

Statistical analysis of the data was carried out using ANOVA and Tukey's

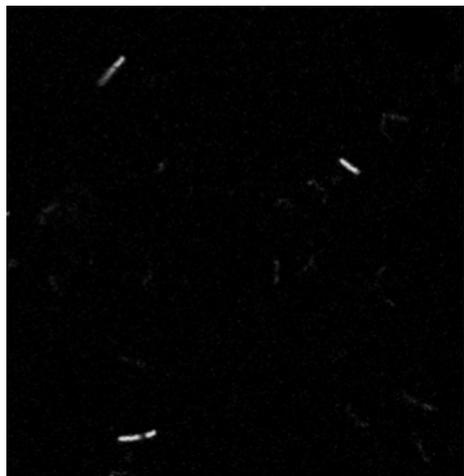


Figure 1. Immunofluorescent staining of *Lactobacillus casei* Shirota. Bacteria + AFB₁ + Ab-FITC interaction shows fluorescent images of bacteria. All images were taken using immersion objectives (100×).

mean comparison tests ($P \leq 0.05$) using the Minitab statistical package version 14.1 to identify significant differences among treatments. All experiments and analyses were carried out in duplicate.

3. RESULTS

Because part of the objectives of this study were to assess the ability of *Lactobacillus casei* Shirota to bind AFB₁ by fluorescent monoclonal antibody staining, and to evaluate the AFB₁-bacteria interaction by AFM, the results shown in this part of the work refer only to images, so numerical data are not presented. Different visual fields were examined in both microscope assays, and according to a good reproducibility of the images, we selected those that were representative of the control and/or treatment groups.

When samples were analyzed by FM no fluorescent staining was observed in visual fields of the micrographs obtained from

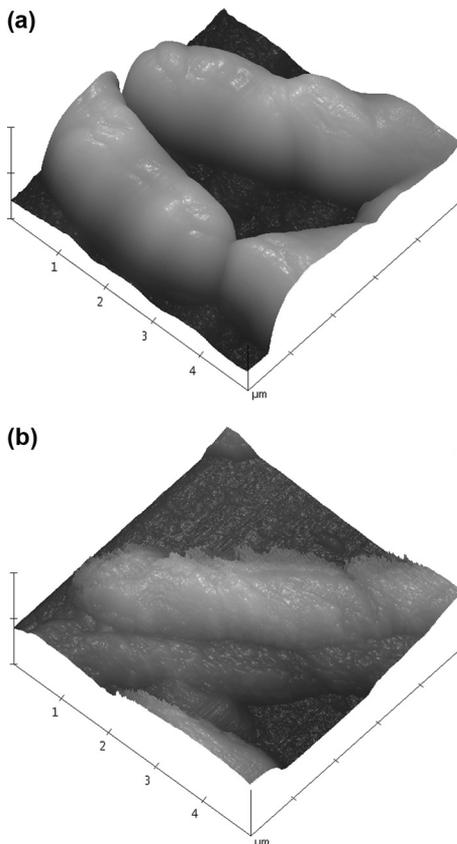


Figure 2. Tapping mode AFM image of *Lactobacillus casei* Shirota. (a) Common morphology of bacteria without exposure to AFB₁. (b) Example of alteration induced in bacterial cell surface by AFB₁ exposure.

bacteria without treatment and both interactions, Bacteria + Ab-FITC and Bacteria + AFB₁. This finding does not only suggest that both bacteria without treatment and aflatoxin bound to bacteria do not show a reactive fluorescent per se, but also that bacteria alone do not possess epitopes recognized by Ab-FITC on their surface. These data could eliminate the possibility of fake results and serve as controls. Otherwise, the micrographs obtained from Bacteria + AFB₁ + Ab-FITC interaction (Fig. 1) showed that

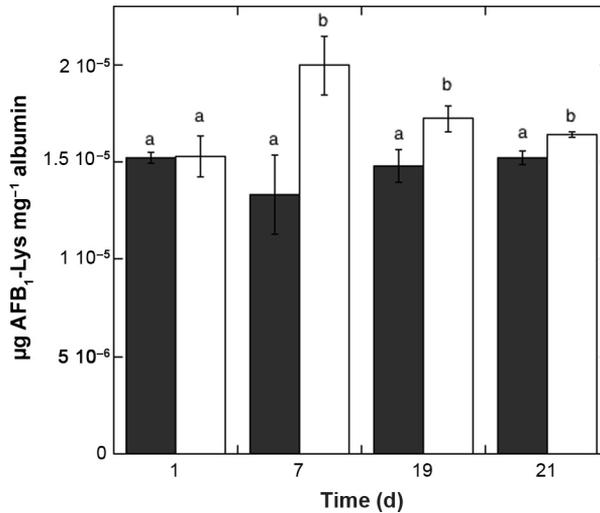


Figure 3. Level of AFB₁-Lys adducts quantified by ELISA in serum samples of rats treated with Bacteria + AFB₁ (■) and treated only with AFB₁ (□). Columns with different letters for each time group are statistically different from one another ($P \leq 0.05$).

the bacterial cell surface emitted a strong and uniform fluorescence, which was generated by the fluorochrome molecule (FITC, fluorescein isothiocyanate) after the excitation of the specimen, which most likely indicates that AFB₁ was bound to the bacterial cell surface and then recognized by the specific FITC-labeled antibody against AFB₁.

On the other hand, AFM assay proved useful for imaging the morphology of bacterial with and without AFB₁ treatment. A representative example of the normal morphology of *Lactobacillus casei* Shirota not exposed to AFB₁ is shown in Figure 2a. Bacteria appeared undamaged and showed a characteristic morphology of bacilli with a periphery that was well defined between cells. Bacteria also displayed a smooth and homogeneous cell surface with no evident features, which indicate that bacteria were not deformed or damaged by the operation conditions of the equipment. Meanwhile, *Lactobacillus casei* Shirota treated with AFB₁ (Fig. 2b) revealed conformational changes, most likely caused by AFB₁ bound

into the cell wall surface. The most significant differences in cell morphology changes were a bacterial surface rather irregular and rough with edges not defined. These findings indicate that AFM has strong potential for study of the structure-relationship of AFB₁-cell surface interaction.

In order to quantify the effect of the supplementation with *Lactobacillus casei* Shirota on absorption of AFB₁, quantitative measurement of AFB₁-Lys adducts in serum samples was performed. The results are given in Figure 3. Data indicated no measurable levels of AFB₁-Lys adducts in serum from rats of the control group. However, the presence of adducts in the serum of rats treated only with AFB₁ evidenced the chronic exposure to the mycotoxin. Moreover, rats previously treated with *Lactobacillus casei* Shirota showed a significant ($P \leq 0.05$) decrease of AFB₁-Lys adducts in serum after the first week of the experimental period. These data strongly suggest that the reduction of AFB₁-Lys adduct in blood samples could be attributable to the ability of

Lactobacillus casei Shirota to bind the AFB₁ at intestinal level under chronic toxin exposure.

4. DISCUSSION

Aflatoxicosis has become a great concern to public health and food industries due to a number of outbreaks caused by foods contaminated with aflatoxins [33]. This fact emphasizes the need to develop strategies for reducing toxic effects of aflatoxins by consumption of contaminated food. This study employed FITC-labeled monoclonal antibody against AFB₁ in a sensitive immunocytochemical technique to stain *Lactobacillus casei* Shirota. The method permitted to identify AFB₁ over the cell surface of the bacteria as a specific receptor for FITC-labeled antibody, which proves in a visual and reliable way the ability of the bacterial strain to bind the AFB₁ and supports conclusions from other studies that specific dairy strains of Lactobacilli can remove aflatoxins from aqueous solution by binding means [5, 13, 15, 16]. The cell wall of Gram-positive bacteria contains a wide variety of structures and serves a multitude of functions, most of which are critical to the viability of the cell [25]. Some of these structures serve as an attachment site for different molecules (e.g. proteins, divalent cations) that interact with the bacterial environment [30]. This makes possible that aflatoxin could be bound by some of these structures. Previous studies suggest that aflatoxin binding is predominantly to the peptidoglycan or the structures closely associated with the peptidoglycan [21]. Furthermore, studies involving hydrophobic interactions [13]; teichoic acids-deficient bacteria, and bacteria treated with a polycation [16], point at the dominant role of teichoic acids in binding mechanism. According to this, aflatoxin bound either to surface teichoic acids and/or another cell wall component could mediate antibody-recognition events, and

this serves as the basis for detection of cell surface molecules by immunofluorescence approach. In this respect, it has been reported that FM techniques can detect surface antigens present in a single cell, and distribution of antigen on surface can be interpreted in the context of cell shape [8]. In spite of the number of cell surface receptors (AFB₁) were not quantified by the protocol presented here, the amount of AFB₁ bound was high enough to be detected using microscopy. This means that the molecules were present at over 1000 sites/cell surface, since below this level, FM is not sensitive enough to allow detection of the surface reaction [39].

In general, AFM images of gram-positive bacteria have shown a surface appearance smooth rather than undulant or wavy [36]. This is consistent with the images displayed by the cell surface of *Lactobacillus casei* Shirota found in this study. In a related work both surface smoothness and roughness of other *Lactobacillus* strains have been reported [31]. The authors of this previous work concluded that heterogeneous (rough and/or patchy) surfaces could consist of either a complex polymeric network or a S-layer covered by polymeric substances adopting extended conformations; meanwhile homogeneous (smooth) surfaces could be an outer layer fully covered by a compact protein layer or a layer of fairly low surface density consisting of a single polymer. According to this, we inferred that teichoic acids chains exposed on the cell surface of *Lactobacillus casei* Shirota are arranged in such a way within the cell wall that do not provide differences in surface texture. One possible explanation for this is that glycan chains lie in the plane of the cell surface, and consequently the teichoic acids chains must permeate deeply into the wall and no discrete layered structure would occur [1]. Moreover, it has not only been reported that teichoic acid conformation may have a significant influence on the physiology of bacteria, but also that some

molecules such as divalent cations, salts, and antibiotics can induce structural changes on the teichoic acid structure [4, 20]. This suggests that changes on surface appearance observed in AFM images after exposure of the bacteria to the aflatoxin could be caused by conformational changes of teichoic acids molecules as a consequence of aflatoxin binding.

Rats supplemented with *Lactobacillus casei* Shirota and given multiple-oral doses of AFB₁ showed a significantly lower level of AFB₁-Lys adducts in serum, as compared to rats treated only with AFB₁, which suggests that reduction of adducts present in their bloodstream was originated by the ability of the bacteria to bind AFB₁ inside the intestinal lumen. Similarly, in an ex vivo study using the chicken duodenum loop technique, probiotic strains of *Lactobacillus rhamnosus* removed as high as 54% (w/w) of the added AFB₁ and reduced intestinal absorption by 73% (w/w) [7]. In related in vitro works, Kankaanpää et al. [19] and Gratz et al. [10] demonstrated that aflatoxin binding to the bacterial surface reduced adhesion properties of *Lactobacillus rhamnosus* strains employed (namely, LGG and LC105), which suggests a facilitated faster excretion of immobilized AFB₁ via fecal route. Additionally, an in vivo single-dose experiment in rats [9] showed that aflatoxin binding by LGG successfully increased fecal excretion of AFB₁; however, controversial results regarding AFB₁-albumin adducts detected in plasma of animal receiving AFB₁ were observed. The mean levels of adducts were on average lower in animal receiving AFB₁ plus LGG than in those receiving only AFB₁, though this was not statistically significant. Potential application of LAB as mycotoxin binders in human foods and animal feeds depends on residence time (strain colonization resistance) and stability of the complex in the GI tract [32]. Hence, this effect could have been originated by the short period of bacteria supplementation (3 days before and

3 days after the single oral toxin dose). Moreover, the biological and clinical importance of bacterial residence time in the gut is becoming increasingly recognized [22]. In our study, the application of LAB during the seven days before the first oral dose of AFB₁ and at specific intervals of the experimental period was at a level proportional to human intake (10^9 – 10^{10} CFU) [26] which would be comparable to that consumed by rats in this work with no adverse effects on general health status. Besides, evidence has shown that *Lactobacillus* supplementation may significantly alter the intestinal microbiota [23]. In this respect, a number of clinical trials have shown the capacity of *Lactobacillus casei* Shirota to survive passage through the GI tract and colonize at physiologically significant level following oral intake [34]. According to this, the oral supplementation of *Lactobacillus casei* Shirota could have displaced the native digestive flora of the rodents modifying the GI community in terms of population levels. Therefore, the observed reduction of AFB₁-Lys adduct in blood samples of rats previously treated with bacteria compared with rats treated only with AFB₁ could be attributable to both, the implantation and the long residence time of *Lactobacillus casei* Shirota in the GI tract.

5. CONCLUSION

The results obtained by microscope techniques in this study may not only contribute to the better understanding of the interaction of cell wall components involved on AFB₁ binding mechanisms, but also our data can provide the basis for microbiological detection of chronic human exposition to aflatoxins through fluorescent bacterial staining. On the other hand, the data obtained from in vivo experiment suggest that a constant supplementation with probiotic bacteria may be of value for decreasing aflatoxin absorption at intestinal level even after

a long period of toxin exposure. Hence, our findings may promote the development of nutritional strategies concerning the prevention of adverse health effects of AFB₁.

Acknowledgments: The authors thank Prof. J.A. Lucey (Department of Food Science) and Prof. T.F. Kuech (Department of Chemical and Biological Engineering) of the University of Wisconsin-Madison, USA, for providing laboratory space and fluorescence microscope and atomic force microscope, respectively. The authors are also grateful to M. del Carmen Estrada-Montoya (Department of Food Technology of Animal Origin) of CIAD, Mexico, for her expert technical assistance.

REFERENCES

- [1] Baddiley J., Mechanism and control of cell wall synthesis in bacteria, *Pure Appl. Chem.* 42 (1975) 417–429.
- [2] Bolognani F., Rumney C.J., Rowland R., Influence of carcinogen binding by lactic acid producing bacteria on tissue distribution and in vivo mutagenicity of dietary carcinogens, *Food Chem. Toxicol.* 35 (1997) 535–545.
- [3] Chapot B., Wild C.P., ELISA for quantification of aflatoxin-albumin adducts and their application to human exposure assessment, in: Van-Warhol M., Velzen D., Bullock G.R. (Eds.), *Techniques in Diagnostic Pathology*, Academic Press, New York, USA, 1991, pp. 135–155.
- [4] Doyle R.J., McDannel M.L., Streips U.N., Birdsell D.C., Young F.E., Polyelectrolyte nature of bacterial teichoic acids, *J. Bacteriol.* 118 (1974) 606–615.
- [5] El-Nezami H., Kankaanpää P., Salminen S., Ahokas J., Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B₁, *Food Chem. Toxicol.* 36 (1998) 321–326.
- [6] El-Nezami H., Kankaanpää P., Salminen S., Ahokas J., Physicochemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media, *J. Food Prot.* 61 (1998) 466–468.
- [7] El-Nezami H., Mykkanen H., Kankaanpää P., Salminen S., Ahokas J., Ability of *Lactobacillus* and *Propionibacterium* strains to remove aflatoxin B₁ from the chicken duodenum, *J. Food Prot.* 63 (2000) 549–552.
- [8] Goldman R.D., Antibodies: indispensable tools for biomedical research, *Trends Biochem. Sci.* 25 (2000) 593–595.
- [9] Gratz S., Taubel M., Juvonen R.O., Viluksela M., Turner P.C., Mykkanen H., El-Nezami H., *Lactobacillus rhamnosus* strain GG modulates intestinal absorption, fecal excretion, and toxicity of aflatoxin B₁ in rats, *Appl. Environ. Microbiol.* 72 (2006) 7398–7400.
- [10] Gratz S., Wu Q.K., El-Nezami H., Juvonen R.O., Mykkanen H., Turner P.C., *Lactobacillus rhamnosus* strain GG reduces aflatoxin B₁ transport, metabolism, and toxicity in Caco-2 cells, *Appl. Environ. Microbiol.* 73 (2007) 3958–3964.
- [11] Groopman J.D., Kensler T.W., Wild C.P., Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries, *Annu. Rev. Public Health* 29 (2008) 187–203.
- [12] Guan S., Ji C., Zhou T., Li J., Ma Q., Niu T., Aflatoxin B₁ degradation by *Stenotrophomonas maltophilia* and other microbes selected using coumarin medium, *Int. J. Mol. Sci.* 9 (2008) 1489–1503.
- [13] Haskard C.A., El-Nezami H.S., Kankaanpää P.E., Salminen S., Ahokas J.T., Surface binding of aflatoxin B₁ by lactic acid bacteria, *Appl. Environ. Microbiol.* 67 (2001) 3086–3091.
- [14] Hayes J.D., Chanas S.A., Henderson C.J., McMahon M., Sun C., Moffat G.J., Wolf C.R., Yamamoto M., The Nrf₂ transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin, *Biochem. Soc. Trans.* 28 (2000) 33–41.
- [15] Hernandez-Mendoza A., Garcia H.S., Steele J.L., Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B₁, *Food Chem. Toxicol.* 47 (2009) 1064–1068.
- [16] Hernandez-Mendoza A., Guzman-de-Pena D., Garcia H.S., Key role of teichoic acids on aflatoxin B₁ binding by probiotic bacteria, *J. Appl. Microbiol.* 107 (2009) 395–403.

- [17] IACUC, Guideline of Selected Techniques for Rat and Mouse Blood Collection, Guideline 9, USA, 1999.
- [18] Kandler O., Weiss N., Genus *Lactobacillus*, in: Sneath P.H.A., Mair N.S., Sharpe M.E., Holt J.G. (Eds.), *Bergey's Manual of Systematic Bacteriology*, Williams & Wilkins, Baltimore, London, Los Angeles, 1984.
- [19] Kankaanpää P., Tuomola E., El-Nezami H., Ahokas J., Salminen S.J., Binding of aflatoxin B₁ alters the adhesion properties of *Lactobacillus rhamnosus* strain GG in a Caco-2 model, *J. Food Prot.* 63 (2000) 412–414.
- [20] Kusser W., Zimmer K., Fiedler F., Characteristics of the binding of aminoglycoside antibiotics to teichoic acids. A potential model system for interaction of aminoglycosides with polyanions, *Eur. J. Biochem.* 151 (1985) 601–605.
- [21] Lahtinen S.J., Haskard C.A., Ouwehand A.C., Salminen S.J., Ahokas J.T., Binding of aflatoxin B₁ to cell wall components of *Lactobacillus rhamnosus* strain GG, *Food Addit. Contam.* 21 (2004) 158–164.
- [22] Lee Y.K., Ho P.S., Low C.S., Arvilommi H., Salminen S., Permanent colonization by *Lactobacillus casei* is hindered by the low rate of cell division in mouse gut, *Appl. Environ. Microbiol.* 70 (2004) 670–674.
- [23] Molly K., De Smet I., Nolet L., Vande Woestyne M., Verstraete W., Effect of *Lactobacilli* on the ecology of the microbiota cultured in the SHIME reactor, *Microb. Ecol. Health Dis.* 9 (1996) 79–89.
- [24] Murugavel K.G., Naranatt P.P., Shankar E.M., Mathews S., Raghuram K., Rajasambandam P., Jayanthi V., Surendran R., Murali A., Srinivas U., Palaniswamy K.R., Srikumari D., Thyagarajan S.P., Prevalence of aflatoxin B₁ in liver biopsies of proven hepatocellular carcinoma in India determined by an in-house immunoperoxidase test, *J. Med. Microbiol.* 56 (2007) 1455–1459.
- [25] Navarre W.W., Schneewind O., Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope, *Microbiol. Mol. Biol. Rev.* 63 (1999) 174–229.
- [26] Orrhage K.M., Annas A., Nord C.E., Brittebo E.B., Rafter J.J., Effects of lactic acid bacteria on the uptake and distribution of the food mutagen Trp-P-2 in mice, *Scand. J. Gastroenterol.* 37 (2002) 215–221.
- [27] Park S., Hwang M., Kim Y., Kim J., Song J., Lee K., Jeong K., Rhee M., Kim K., Kim T., Comparison of pH and bile resistance of *Lactobacillus acidophilus* strains isolated from rat, pig, chicken, and human sources, *World J. Microbiol. Biotechnol.* 22 (2006) 35–37.
- [28] Phillips T.D., Dietary clay in the chemoprevention of aflatoxin-induced disease, *Toxicol. Sci.* 52 (1999) 118–126.
- [29] Pringle J.R., Preston R.A., Adams A.E., Stearns T., Drubin D.G., Haarer B.K., Jones E.W., Fluorescence microscopy methods for yeast, *Methods Cell Biol.* 31 (1989) 357–435.
- [30] Sara M., Conserved anchoring mechanisms between crystalline cell surface S-layer proteins and secondary cell wall polymers in Gram-positive bacteria?, *Trends Microbiol.* 9 (2001) 47–49.
- [31] Schar-Zammaretti P., Ubbink J., The cell wall of lactic acid bacteria: surface constituents and macromolecular conformations, *Biophys J.* 85 (2003) 4076–4092.
- [32] Shetty P.H., Jespersen L., *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents, *Trends Food Sci. Technol.* 17 (2006) 48–55.
- [33] Strosnider H., Azziz-Baumgartner E., Banziger M., Bhat R.V., Breiman R., Brune M.N., DeCock K., Dilley A., Groopman J., Hell K., Henry S.H., Jeffers D., Jolly C., Jolly P., Kibata G.N., Lewis L., Liu X., Lubner G., McCoy L., Mensah P., Miraglia M., Misore A., Njapau H., Ong C.N., Onsongo M.T., Page S.W., Park D., Patel M., Phillips T., Pineiro M., Pronczuk J., Rogers H.S., Rubin C., Sabino M., Schaafsma A., Shephard G., Stroka J., Wild C., Williams J.T., Wilson D., Workgroup report: public health strategies for reducing aflatoxin exposure in developing countries, *Environ. Health Perspect.* 114 (2006) 1898–1903.
- [34] Tuohy K.M., Pinart-Gilberga M., Jones M., Hoyles L., McCartney A.L., Gibson G.R., Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora, *J. Appl. Microbiol.* 102 (2007) 1026–1032.
- [35] Turbic A., Ahokas T., Haskard C.A., Selective in vitro binding of dietary mutagens, individually or in combination, by lactic acid

- bacteria, *Food Addit. Contam.* 19 (2002) 144–152.
- [36] Umeda A., Saito M., Amako K., Surface characteristics of Gram-negative and Gram-positive bacteria in an atomic force microscope image, *Microbiol. Immunol.* 42 (1998) 159–164.
- [37] Vinderola C.G., Ballo N., Reinheimer J.A., Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage, *Food Res. Int.* 33 (2000) 97–102.
- [38] Williams J.H., Phillips T.D., Jolly P.E., Stiles J.K., Jolly C.M., Aggarwal D., Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions, *Am. J. Clin. Nutr.* 80 (2004) 1106–1122.
- [39] Willingham M.C., Fluorescence labeling of surface antigens of attached or suspended tissue-culture cells, in: Javols L.C. (Ed.), *Methods in Molecular Biology*, Human Press Inc., Totowa, USA, 1994, pp. 113–119.