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Note

In vivo assessment of the potential protective effect of *Lactobacillus casei* Shirota against aflatoxin B_1

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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 B_1 (AFB_1) by fluorescent monoclonal antibody staining, (2) to evaluate the AFB_1-bacteria interaction by atomic force microscopy, and (3) to determine its effect on intestinal absorption through detection of AFB_1-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 AFB_1 into the bacterial cell envelope. The images also revealed that aflatoxin binding produces structural changes that modify the bacterial cell surface. AFB_1-Lys adducts quantified from blood samples were found to be present at significantly lower levels in animals receiving AFB_1 plus bacteria than in those receiving only AFB_1. 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 B_1 / cell surface / *Lactobacillus casei* Shirota

摘要 – 体内测定 *Lactobacillus casei* Shirota 菌株对黄曲霉毒素 B_1 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 B_1 (AFB_1) 的粘附作用; (2) 采用原子力显微镜评价 AFB_1-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 AFB_1-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 AFB_1 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 AFB_1-Lys 络合物的定量测定，黄曲霉

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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 AFB_1-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 B_1 / 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 B_1. 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 B_1 (AFB_1) par marquage à un anticorps monoclonal fluorescent, (2) d'évaluer l'interaction bactérie-AFB_1 par microscopie à force atomique, (3) de déterminer ses effets sur l'absorption intestinale par la détection des composés AFB_1-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 AFB_1 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 AFB_1-Lys quantifiés à partir des échantillons sanguins étaient présents à des niveaux significativement plus faibles chez les animaux recevant AFB_1 plus bactérie par rapport à ceux recevant seulement AFB_1. 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 B_1 / 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 B_1 (AFB_1) 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, chlorophylin) 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 AFB_1 has greatly increased. Several fungal species have been found to be able to transform AFB_1 into less toxic metabolites. Reduction of AFB_1 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 AFB_1 removal. Approximately a minimum of 2–5 × 10^9 CFU·mL^{-1} is required for significant AFB_1 removal (13–50%), while a concentration of 2 × 10^{10} CFU·mL^{-1} is capable of reducing the AFB_1 level to < 0.1% and 13% [2, 5]. The effect of various AFB_1 concentrations on AFB_1 removal has also been tested. The amount of AFB_1 removed increased with increasing concentration of AFB_1 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 AFB_1 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 AFB_1 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 AFB_1 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 AFB_1 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 AFB_1 by fluorescent monoclonal antibody staining, to evaluate the AFB_1-bacteria interaction by atomic force microscopy (AFM), and to determine its effect on intestinal absorption through detection of AFB_1-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 AFB_1 by fluorescence microscopy (FM), four experimental procedures using either FITC-labeled monoclonal antibody against AFB_1 (Ab-FITC (HyTest Ltd., Turku, Finland)), Bacteria, and/or AFB_1 were proposed as follows: (1) Bacteria, (2) Bacteria + Ab-FITC, (3) Bacteria + AFB_1, and (4) Bacteria + AFB_1 + Ab-FITC. Prior to labeling working cells, AFB_1 (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 × 10^9 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 AFB_1 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 AFB_1 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. *AFB_1-Bacteria interaction assay by AFM*

To evaluate the AFB_1-*Lactobacillus casei* Shirota interaction, both bacteria with and without AFB_1 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 mg·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 AFB_1 absorption at intestinal level under toxin exposure

For in vivo assay, 15 male Wistar rats (200 ± 20 g) were obtained from Harlan Teklad Inc. (Mexico City) and were housed individually in stainless cages under controlled temperature (21 ± 1 °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 AFB_1) 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 AFB_1 (4.0 mg·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 AFB_1 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 AFB_1 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 AFB_1-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 μg·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 AFB_1-Lys·mg^{-1} albumin.

2.4. Statistical analysis

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

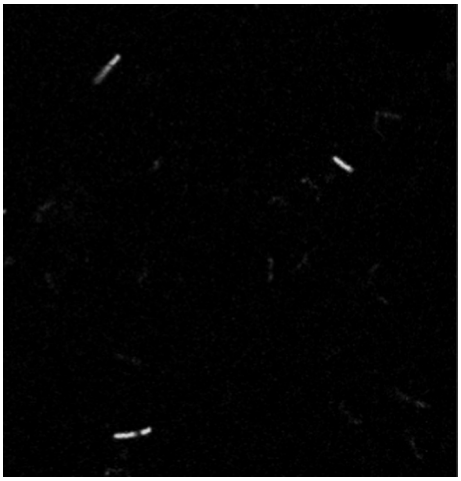

Figure 1. Immunofluorescent staining of *Lactobacillus casei* Shirota. Bacteria + AFB_1 + Ab-FITC interaction shows fluorescent images of bacteria. All images were taken using immersion objectives (100×).

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 AFB_1 by fluorescent monoclonal antibody staining, and to evaluate the AFB_1-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

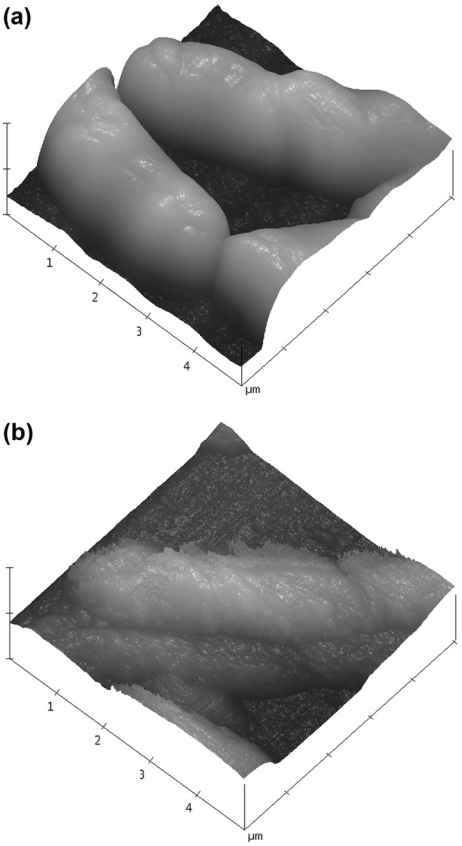

Figure 2. Tapping mode AFM image of *Lactobacillus casei* Shirota. (a) Common morphology of bacteria without exposure to AFB_1. (b) Example of alteration induced in bacterial cell surface by AFB_1 exposure.

bacteria without treatment and both interactions, Bacteria + Ab-FITC and Bacteria + AFB_1. 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 + AFB_1 + Ab-FITC interaction (Fig. 1) showed that

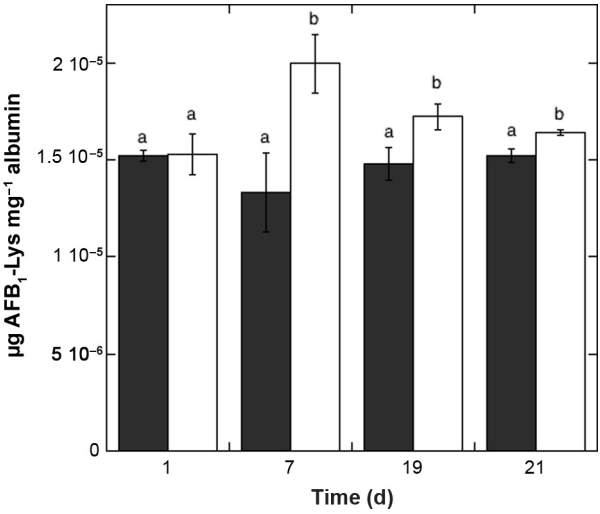

Figure 3. Level of AFB_1-Lys adducts quantified by ELISA in serum samples of rats treated with Bacteria + AFB_1 (■) and treated only with AFB_1 (□). Columns with different letters for each time group are statistically different from one another ($P \leq 0.05$).

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 AFB_1 was bound to the bacterial cell surface and then recognized by the specific FITC-labeled antibody against AFB_1.

On the other hand, AFM assay proved useful for imaging the morphology of bacterial with and without AFB_1 treatment. A representative example of the normal morphology of *Lactobacillus casei* Shirota not exposed to AFB_1 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 AFB_1 (Fig. 2b) revealed conformational changes, most likely caused by AFB_1 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 AFB_1-cell surface interaction.

In order to quantify the effect of the supplementation with *Lactobacillus casei* Shirota on absorption of AFB_1, quantitative measurement of AFB_1-Lys adducts in serum samples was performed. The results are given in Figure 3. Data indicated no measurable levels of AFB_1-Lys adducts in serum from rats of the control group. However, the presence of adducts in the serum of rats treated only with AFB_1 evidenced the chronic exposure to the mycotoxin. Moreover, rats previously treated with *Lactobacillus casei* Shirota showed a significant ($P \leq 0.05$) decrease of AFB_1-Lys adducts in serum after the first week of the experimental period. These data strongly suggest that the reduction of AFB_1-Lys adduct in blood samples could be attributable to the ability of

Lactobacillus casei Shirota to bind the AFB_1 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 AFB_1 in a sensitive immunocytochemical technique to stain *Lactobacillus casei* Shirota. The method permitted to identify AFB_1 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 AFB_1 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 (AFB_1) were not quantified by the protocol presented here, the amount of AFB_1 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 AFB_1 showed a significantly lower level of AFB_1-Lys adducts in serum, as compared to rats treated only with AFB_1, which suggests that reduction of adducts present in their bloodstream was originated by the ability of the bacteria to bind AFB_1 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 AFB_1 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 AFB_1 via fecal route. Additionally, an in vivo single-dose experiment in rats [9] showed that aflatoxin binding by LGG successfully increased fecal excretion of AFB_1; however, controversial results regarding AFB_1-albumin adducts detected in plasma of animal receiving AFB_1 were observed. The mean levels of adducts were on average lower in animal receiving AFB_1 plus LGG than in those receiving only AFB_1, 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 AFB_1 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 AFB_1-Lys adduct in blood samples of rats previously treated with bacteria compared with rats treated only with AFB_1 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 AFB_1 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 AFB_1.

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