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In vitro assessment of *L.acidophillius* and *L.casei* binding capacity against aflatoxins B1

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Abstract

The aim of this study was to investigate the efficacy of *Lactobacillus acidophilus* and *Lactobacillus casei* for the binding of aflatoxin B1 *in vitro* in aqueous solution. We have chosen to focus on those bacteria due to their long history of use as a probiotic, the availability of a bank of strains with documented genetic diversity isolated from various ecological niches, and the availability of genetic tools to examine mechanisms of probiotic activity. The *Lactobacillus acidophilus* exhibited different degrees of aflatoxin B1 binding, the highest AFB1 binding was *L. acidophilus* 20079, which bound 89.4% in probiotic condition and 87.14% in prebiotic condition of the available AFB1 (10 ppb) after 4h of incubation time at pH6.0. The *L. casei* 20011 bound 82.87% in probiotic condition and 81.24% in prebiotic condition after 4 h of incubation time at pH6.0. The incubation time (2 and 4 h) affect the elimination of the toxin as significant differences in the amount of AFB1 removed by *L. casei* and *L. acidophilus* were observed.

Introduction

Mycotoxins are secondary metabolites of moulds that can be toxic to humans or animals upon ingestion. These compounds are structurally diverse and are capable of causing a variety of well-characterized biological and toxicological effects [1]. Severity, which depends on the toxicity of the mycotoxins, the extent of exposure, age and nutritional status of the individual and possible synergistic effects of other chemical structure of mycotoxins vary considerably [2]. Based on the observation that marked immunosuppression is a frequently observed effect in field cases of low-level mycotoxins exposure in livestock [3].

Aflatoxin (AF) is a mycotoxin produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin is cutely toxic, immunosuppressive, and hepatotoxic in young broiler chickens [4]. Aflatoxin is the most toxic and carcinogenic among the known mycotoxins [5]. It has been shown to be mutagenic, genotoxic, and hepatocarcinogenic [6]. Because of these effects, aflatoxins are regulated in the low parts per billion range in diets in most developed countries [7].

Practical methods to detoxify mycotoxin-contaminated grain on large scale and in cost-effective manner are not currently available. A variety of physical, chemical, and biological techniques have been employed, however, they have met with only limited success [8]. Recent approach has been the addition of probiotics or prebiotics such as lactic acid bacteria and some other bacteria or fungi [9].The protective role of microbial against gastrointestinal toxicogenic and pathogens and the underlying mechanisms have received special attention as such interaction has served as one criterion for selecting new probiotics for human use [10]. The mechanisms by which probiotics exert their beneficial effects on the host are largely unknown [11].Among the identified mechanisms are the reduction of luminal pH, competition with pathogens for adhesion sites and nutritional sources, secretion of antimicrobial substances, toxin inactivation, and immune stimulation [10]. Most of the approaches have not been adopted due to high costs, loss of nutritional and sensory properties of the products, or practical difficulties involved in detoxification process [12]. Therefore, a promising alternative is the use of microorganisms as FB1 sequestering agents. Inclusion of such microbes in the diet may reduce the toxic effects of mycotoxins on humans, as a FB1–microorganism complex may decrease availability of the mycotoxin and consequently its absorption in the gastrointestinal tract [13].

[14] have used *Saccharomyces cerevisiae* and lactic acid bacteria (LAB) as probiotics and potential mycotoxin decontaminating microorganisms because of their ability to bind these toxic metabolites. Nevertheless, despite several publications having reported in vitro binding by LAB and yeast strains of mycotoxins such as aflatoxin B1 (AFB1) [15,16,17], zearalenone and certain trichothecenes [18,19] little is known about the mechanism involved in

aflatoxin removal. An understanding of the binding mechanism is required to allow the optimization and safe dietary application of this methodology [20, 21].

A focus in our research group is the development of probiotic or prebiotic strains of *Lactobacillus casei* and *Lactobacillus acidophillius* ability to bind AFB1 could decrease the bioavailability of these compounds and limit their toxic effects on human and animals.

Materials and methods

This study was carried out at School of Agriculture, Newcastle University-Newcastle upon tyne / England from (12 Aug 2014) to (12 Nov 2014).

Bacterial strains, growth medium and cultural conditions.

Lactobacillus acidophilus (20079, Type strain) and *Lactobacillus casei* (20011, Type strain) from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures , was grown on De Man, Rogosa, Sharpe (MRS) broth under microaerophilic conditions for 24 h at 37 °C without shaking, using inoculum at 0.1% (v/v) from an overnight culture at 37 C° . Cell suspension concentration was determined using a hemocytometer (Bright-Line™ Cambridge Instruments, Inc. England). Viability was confirmed by standard count methods using MRS. Cells were removed by centrifugation at 10,000 g for 5 min, washed three times and re-suspended in sterile 0.5% NaCl solution to final concentration of 1.5×10^7 viable bacterial cells per ml.

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 during20 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 7.4 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 [22], and results were expressed as colony forming units per milliliter (CFU/mL).

AFB1 binding assay.

The AFB1 binding assay was performed according to [15]. Stock solution of solid AFB1 (Sigma-Aldrich™, Germany) was suspended in benzene–acetonitrile (97:3 v/v) to obtain an AFB1 concentration of 10 ppb. The benzene–acetonitrile was evaporated by heating at 70 C° for 10 min, and 50 µl of methanol was added and brought to final volume with PBS, pH 7.4. Cells were washed twice with PBS and incubated at 37 C° for 30 min in a shaking bath with 1 ml of PBS containing AFB1 (10 ppb). Then, cells were pelleted by centrifugation at 5000 g for 15 min at room temperature, and the supernatant containing unbound AFB1 was collected and stored at −20 C° until ELISA test.

Determining the residual AFB1 through ELISA test.

Aflatoxins were determined by a monoclonal antibody-based ic-ELISA by using Aflatoxin ELISA Test Kits (Helica Biosystems, Inc. Santa Ana,CA 92704,USA) sensitivity: 0.1 ppb and as the product protocol procedure .

After exposing the PBS solutions which contained bacterial strains of LAB and AFB1, the solutions were centrifuged for 15 min at 10 000 g/min. The bacteria released residues. For the following step, quantities of 500 µ L from the upper portions of the samples were removed. For purpose of gaining a greater assurance about the removal of all the bacteria, all the solutions were centrifuged under the previous conditions and quantities of 200 µL from the upper portions of the solutions were removed once again.

Results.

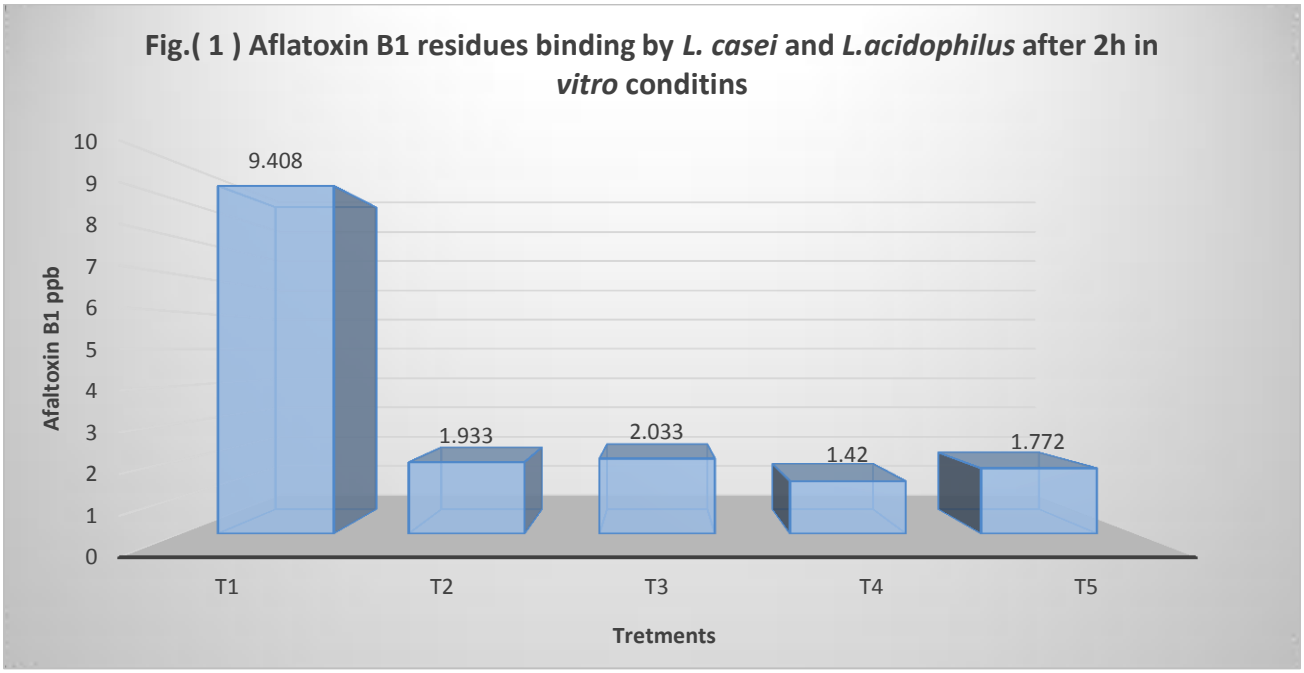
In order to establish the mechanism of the removal of AFB1 (Fig. 1) shows AFB1 binding by *L. casei* and *L. acidophilus* from liquid medium, assayed at toxin concentration. Both microorganisms were able to bind the mycotoxin and the level of binding appeared to vary between the species indicating the microorganism specific nature of binding. The percentage of AFB1 removed it was similar at all concentration tested. To find out which were able to bind the mycotoxin and the level of binding appeared to vary between the species indicating the microorganism specific nature of binding tow experiments conducted in different time.

The differences in binding ability between probiotic and prebiotic statically not significant ($p \leq 0.05$) in all assays, *L. casei* probiotic result found to bind (80.06%) of AFB1 in pH (6.0) compare with (79.6%) in use *L. casei* probiotic condition.

Prebiotic of *L. acidophilus* showed a higher binding result (82.23%) than probiotic of *L. acidophilus* (80.58%) at pH (6.0) in the testing solution.

[23] assess the ability of five probiotic bacteria to bind aflatoxin B1 in order to understand the binding process, protoplasts, spheroplasts and cell wall components of two strains were analyzed to assess their capacity to bind AFB1 *Lactobacillus reuteri* strain NRRL14171 and *Lactobacillus casei* strain Shirota were the most efficient strains for binding AFB1.

The incubation time (2 and 4 h) affect the elimination of the toxin as significant differences in the amount of AFB1 removed by *L. casei* and *L. acidophilus* were observed, the results showed that *L. acidophilus* probiotic (89.4%) and prebiotic (87.14%) (heat treatment) increase their AFB1 binding capacity and as a general trend, the extent of removal of AFB1 increased with extended incubation time ($p \leq 0.05$) at 4 h , independently on cell viability, for both strains of bacteria. However, in contrast to AFB1 removal, *L. casei* probiotic (82.87%) and prebiotic (81.24%) reduction continuously depending on incubation time for probiotic and prebiotic of both strains.



T1 Aflatoxin 10 ppb (PBS).

T2 Aflatoxin 10 ppb (PBS) + *L. casei* 1.5×10^7 CFU/mL (PBS) live (probiotic).

T3 Aflatoxin 10 ppb (PBS) + *L. casei* 1.5×10^7 CFU/mL (PBS) dead (prebiotic).

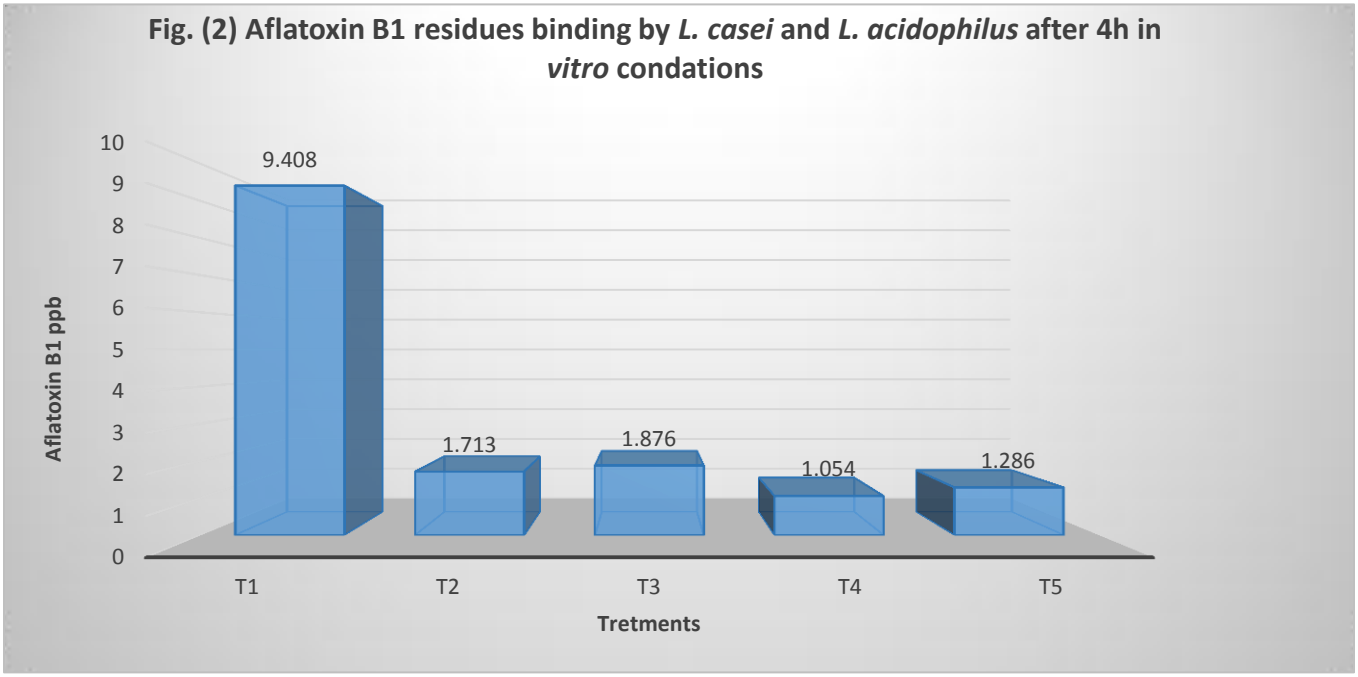
T4 Aflatoxin 10 ppb(PBS) + *L. acidophilus* 1.5×10^7 CFU/mL (PBS) live (probiotic).

T5 Aflatoxin 10 ppb(PBS) + *L. acidophilus* 1.5×10^7 CFU/mL (PBS) dead (prebiotic).

It has been proposed that the consumption of lactic acid bacteria capable of binding or degrading foodborne carcinogens would reduce human exposure to these deleterious compounds. The ability of eight strains of *Lactobacillus casei* to bind aflatoxin B1 in aqueous solution was investigated. The strain with the highest AFB1 binding was *L. casei* L30, which bound 49.2% of the available aflatoxin (4.6l g/mL) [24].

Mixed cultures of the bacteria were investigated. *Lactobacillus plantarum* and *Lactococcus lactis* were separately able to detoxify aflatoxin B1 in solutions. *L. Plantarum* had a better detoxification rate (46%)

than *Lc. Lactis* (27%). When *L. Lactis* and *L. plantarum* were incubated in separate tubes and then mixed, that group had a significantly increased ability to bind toxins (59%) compared to their use alone. When these strains were incubated together in a single broth culture, the most successful detoxification rate (81%) was achieved [25].



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T4 Aflatoxin 10 ppb (PBS) + *L. acidophilus* 1.5×10^7 CFU/mL (PBS) live (probiotic).

T5 Aflatoxin 10 ppb (PBS) + *L. acidophilus* 1.5×10^7 CFU/mL (PBS) dead (prebiotic).

Our findings confirm that probiotic bacteria could act as biological barriers in normal intestinal conditions there by reducing the bioavailability of AFB1 ingested orally in a single or multiple doses, thus avoiding its toxic effects. In previous result we demonstrated that *Lactobacillus casei* and *Lactobacillus acidophilus* showed high efficiency in AFB1 removal from liquid media.

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