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Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B_1

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ABSTRACT

It has been proposed that the consumption of lactic acid bacteria capable of binding or degrading food-borne carcinogens would reduce human exposure to these deleterious compounds. In the present study, the ability of eight strains of *Lactobacillus casei* to bind aflatoxin B_1 in aqueous solution was investigated. Additionally, the effect of addition of bile salts to the growth medium on aflatoxin B_1 binding was assessed. The eight strains tested were obtained from different ecological niches (cheese, corn silage, human feces, fermented beverage). The strains exhibited different degrees of aflatoxin binding; the strain with the highest AFB_1 binding was *L. casei* L30, which bound 49.2% of the available aflatoxin (4.6 μg/mL). In general, the human isolates bound the most aflatoxin B_1 and the cheese isolates the least. Stability of the bacterial–aflatoxin complex was assessed by repeated washings. Binding was to a limited degree (0.6–9.2% release) reversible; the *L. casei* 7R1–aflatoxin B_1 complex exhibited the greatest stability. *L. casei* L30, a human isolate, was the strain least sensitive to the inhibitory effects of bile salts. Exposure of the bacterial cells to bile significant increased aflatoxin B_1 binding and the differences between the strains was reduced.

1. Introduction

The human diet contains a wide variety of natural carcinogens that are present in foods as the result of contaminated raw materials or produced during the processing and/or cooking of foods. Aflatoxins (AFs), a group of potent mycotoxins with mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive properties, are of particular importance because of their adverse effects on animal and human health (Lewis et al., 2005). AFs are produced as secondary metabolites of fungal strains (*Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius*) that grow on a variety of food and feed commodities during growth, harvest, storage, and transportation of these products (Peltonen et al., 2001; Jiang et al., 2005). Aflatoxin B_1 (AFB_1), the most toxic AF, is of particular interest because it is a frequent contaminant of many food products and one of the most potent naturally occurring mutagens and carcinogens known (Teniola et al., 2005).

Concerns related to the negative health impacts of AFs have lead to the investigation of strategies to prevent their formation in foods, as well as, to eliminate, inactivate or reduce the bioavailability of these toxins in contaminated products. Techniques to eliminate, inactivate or reduce the bioavailability of AFs include physical, chemical, and biological methods. Physical methods include manually sorting out of contaminated grains (detected by fluorescence) and removal of contaminated grains or kernels via flotation. These processes have at least two drawbacks: (1) high cost of removing and disposing of the contaminated materials, and (2) difficulty achieving complete removal contaminated materials without wasting significant portions of uncontaminated product (Yiannikouris and Jouany, 2002). Chemical methods, which alter the chemical structure of AFs by a chemical treatment (e.g. ammonia gas or ammonium hydroxide, 0.10% sodium hydroxide solution liquid, propionic acid solution bubbled with sulfur dioxide gas), or by physical absorption onto a reactive substrate (e.g. phyllosilicate clay) have been used to convert AFs to less toxic and mutagenic compounds or to immobilize them (Méndez-Albores et al., 2007). Limitations such as loss of product nutritional and sensory qualities, as well as, the expensive equipment required for these techniques has encouraged the recent emphasis on biological methods (Teniola et al., 2005).

Lactic acid bacteria (LAB) and bifidobacteria, due in large part to their GRAS status and use as probiotics, are of particular interest for reducing the bioavailability of AFs. A number of studies have screened these microorganisms for the ability to bind to AFs and have reported a wide range of genus, species and strain specific binding capacities (Bolognani et al., 1997; El-Nezami et al., 1998b; Peltonen et al., 2000, 2001; Haskard et al., 2001; Lee et al., 2003; Hwang et al., 2005; Zinedine et al., 2005; Shahin, 2007). The bacterial cell envelope appears to be the site of AF binding, with cell wall polysaccharides and peptidoglycan thought be

Abbreviations: AFB_1, aflatoxin B_1; AFs, aflatoxins; CFU/mL, colony-forming units per milliliters; LAB, lactic acid bacteria; PBS, phosphate-buffer saline.

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the molecules of greatest importance (Peltonen et al., 2001; Haskard et al., 2001; Lahtinen et al., 2004). The stability of the AF–bacterial cell complex is also a key consideration when evaluating a strains ability to reduce AF bioavailability in foods, as AF release during gastric passage would have clear negative health implications. Binding of AF to bacterial cells has been demonstrated to be reversible and the stability of the AF–bacterial cell complex dependent on the strain utilized, conditions utilized during complex formation and the treatment used to assess stability (Haskard et al., 2001). The conditions of particular interest are low pH and the presence of bile, as they are important environmental stresses that bacterial cells encounter during passage through the gastrointestinal tract (GI). While the influence of low pH on AF binding has been evaluated (Haskard et al., 2000, 2001), we are unaware of any studies that have evaluated the influence of bile on AF binding. The use of LAB and bifidobacteria to reduced human exposure to AFs holds great promise, however additional studies are required to precisely define the AF binding site(s), as well as GI relevant conditions that effect AF binding and the stability of the AF–bacterial cell complex.

A focus in our research group is the development of probiotic strains of *Lactobacillus casei*. We have chosen to focus on *L. casei* due to its 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 objectives of this study were to examine a bank of strains of *L. casei* from different ecological niches for the ability to stably bind AFB_1 and the effect of bile on AFB_1 binding.

2. Materials and methods

2.1. Bacterial strains, growth medium and cultural conditions

L. casei strains were obtained from Dr. Steele's culture collection at the University of Wisconsin, Department of Food Science (Madison, WI, USA). All of the strains utilized were confirmed as *L. casei* by 16S sequencing and have been previously described by Cai et al. (2007). The strains and their ecological niches of isolation are listed in Table 1. Stock cultures were maintained at −80 °C in 20% (v/v) glycerol. Working cultures were prepared from frozen stocks by two transfers in MRS broth (De Man, Rogosa & Sharpe, Difco™) with inoculations at 0.3% (v/v) and static incubation at 37 °C for 12 and 8 h, respectively.

To conduct AFB_1-binding assays, 1% inoculations were made from the working cultures into 30 mL of MRS broth and incubated statically for 20 h at 37 °C. After incubation, cells were collected by centrifugation (5000 rpm, 10 min, 10 °C) and washed twice with phosphate-buffered saline (PBS, pH 7.2) and once with sterile double-distilled H_2O. Finally, the bacterial pellets were suspended in 20 mL of sterile double-distilled H_2O prior to use in the AFB_1-binding assays. Additionally, the bacterial cells (2–3 × 10^9 CFU/mL) were enumerated using the pour-plate method (Vinderola et al., 2000) and the results are expressed as colony-forming units per milliliter (CFU/mL).

To determine the effect of exposure to bile salts on growth, survival, and AFB_1 binding of *L. casei* strains, an aliquot (1%) of each strain was incubated for 20 h at 37 °C in MRS broth (30 mL) containing 0.05, 0.10, or 0.15% (w/v) bile salts (Sigma Chemical Co., St. Louis, MO, USA). After incubation, cells were harvested, used in the AFB_1-binding assay, and enumerated as described above.

Table 1
L. casei strains examined in this study.

Strain	Code	Origin
L. casei ATCC 334	334	Swiss-type cheese, USA
L. casei L9	L9	Human GI tract, USA
L. casei L30	L30	Human GI tract, USA
L. casei 12A	12A	Corn silage, WI, USA
L. casei 21/1	21/1	Corn silage, WI, USA
L. casei 7R1	7R1	Cheese, Denmark
L. casei DPC 3968	DPC	Cheese, Ireland
L. casei Shirota	LcS	Yakult, Japan

2.2. Preparation of AFB_1 working solution

AFB_1 (Sigma) was dissolved in benzene-acetonitrile (93:7, v/v) to obtain an approximate concentration of 2 mg/mL (Haskard et al., 2001). To prepare a working solution of 4.6 μg/mL, PBS was added directly and the benzene-acetonitrile was evaporated by heating in a water bath at 80 °C for 10 min. An UV–visible spectrum (SmartSpec™ Plus spectrophotometer, BioRad) from a sample of the solution was recorded, and the actual concentration was calculated from the Lambert–Beer equation ($A = \varepsilon \cdot c \cdot l$) using the absorbance at 354 nm and ε_{354} = 19,800 M^{-1} cm^{-1}. The resulting solution was transferred to a glass bottle and stored in the dark at 4 °C until used (Zinedine et al., 2005).

2.3. AFB_1 binding assay

The binding assay utilized was similar to the procedure reported by Peltonen et al. (2001). One milliliter of each active culture (suspended in 20 mL of sterile double-distilled H_2O) was centrifuged (5000 rpm, 10 min, 10 °C), the bacterial pellet was washed twice with 1 mL of sterile double-distilled H_2O, and then suspended in 1.5 mL of the working solution of AFB_1 and incubated for 4 h at 37 °C. Subsequently, cells were removed by centrifugation (5000 rpm, 10 min, 10 °C) and supernatant fluid containing residual AFB_1 collected and analyzed by HPLC. For each strain, a bacterial control (bacteria suspended in PBS) and an AFB_1 control (working solution of AFB_1 without bacteria) were also incubated.

To determine the stability of the bacterial–AFB_1 complex, the complex was washed three times and the amount of released AFB_1 determined. The bacterial–AFB_1 complex was washed by suspending in PBS (1.5 mL), vortexed for 15 s. and incubating at room temperature for 5 min. Then, the bacterial cells were centrifuged and the supernatant collected for quantification of released AFB_1. This procedure was replicated two additional times.

2.4. Quantification of unbound AFB_1 by HPLC

Quantification of AFB_1 in supernatants was conduced by a reverse-phase method with no extraction step, essentially as described by Peltonen et al. (2001). The HPLC system (Hewlett Packard series 1100) consisted of a pump solvent delivery system (Quat-pump, G1322A), a model G1322A degasser, a fluorescence detector (model F1000, Anspec Company, Inc.), a Discovery® C18 column (25 cm × 4.6 mm, 5 μm, Supelco) and a model SP4270 integrator (Spectra-Physics) for data acquisition. A 20 μL sample was injected via an autoinjector (ALS, G1329A). Microfiltered methanol-acetonitrile (60:40 v/v) was used as isocratic mobile phase with a flow rate of 1 mL/min at room temperature. Aflatoxin detection was accomplished by fluorescence with excitation and emission wavelengths of 362 nm and 420 nm, respectively. Retention time of AFB_1 was approximately 4.7 min. The percentage of AFB_1 bound by the bacterial suspension was calculated using the following formula:

$$\%\ AFB_1 = \left[1 - \left(\frac{AFB_1 \text{ peak area of sample}}{AFB_1 \text{ peak area of toxin control}}\right)\right] \times 100 \quad (1)$$

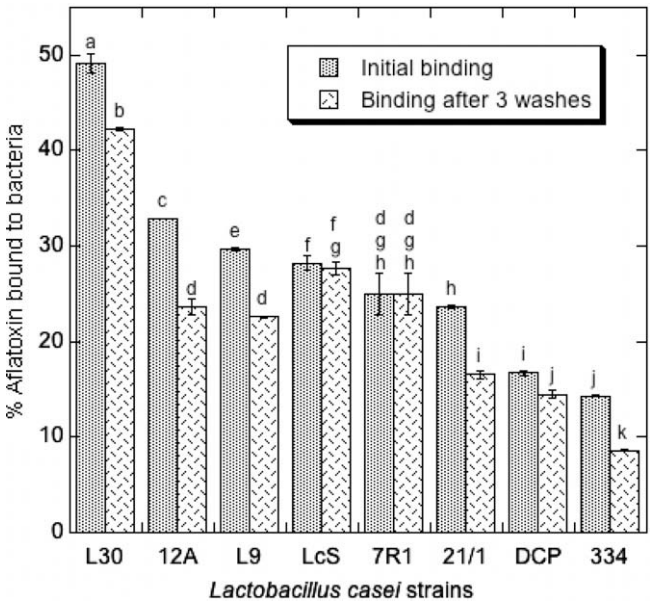

Fig. 1. Percentage of aflatoxin B_1 bound by *Lactobacillus casei* strains (AFB_1 4.6 μg/mL, 4 h at 37 °C) and remaining bound after three washes. Treatments with different letters in each column are statistically different by each bacteria strain ($p \leq 0.05$).