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Surface Binding of Aflatoxin B_1 by Lactic Acid Bacteria

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Specific lactic acid bacterial strains remove toxins from liquid media by physical binding. The stability of the aflatoxin B_1 complexes formed with 12 bacterial strains in both viable and nonviable (heat- or acid-treated) forms was assessed by repetitive aqueous extraction. By the fifth extraction, up to 71% of the total aflatoxin B_1 remained bound. Nonviable bacteria retained the highest amount of aflatoxin B_1. *Lactobacillus rhamnosus* strain GG (ATCC 53103) and *L. rhamnosus* strain LC-705 (DSM 7061) removed aflatoxin B_1 from solution most efficiently and were selected for further study. The accessibility of bound aflatoxin B_1 to an antibody in an indirect competitive inhibition enzyme-linked immunosorbent assay suggests that surface components of these bacteria are involved in binding. Further evidence is the recovery of around 90% of the bound aflatoxin from the bacteria by solvent extraction. Autoclaving and sonication did not release any detectable aflatoxin B_1. Variation in temperature (4 to 37°C) and pH (2 to 10) did not have any significant effect on the amount of aflatoxin B_1 released. Binding of aflatoxin B_1 appears to be predominantly extracellular for viable and heat-treated bacteria. Acid treatment may permit intracellular binding. In all cases, binding is of a reversible nature, but the stability of the complexes formed depends on strain, treatment, and environmental conditions.

Food contaminants entering the body through the oral route are directly exposed to the action of gut microflora. Normal healthy intestinal microflora contains many strains of lactic acid bacteria (LAB), some of which have been isolated, ascribed health benefits, and termed probiotic strains (22). The protective effect of LAB against food mutagens such as heterocyclic amines, *N*-nitroso compounds, and aflatoxins has been reported (8, 12, 19, 24, 27). Many of these studies have involved *Lactobacillus* strains, and physical binding has been proposed as one mechanism of mutagen removal.

This study focuses on the nature of the binding of aflatoxin B_1 (AFB_1) by 12 LAB strains. The potent mycotoxin AFB_1 is a secondary metabolite of *Aspergillus* fungi that grow on a variety of food and feed commodities at any stage during growth, harvest, storage, and transportation. The occurrence of aflatoxin contamination is global, with severe problems especially prevalent in developing countries (11). Aflatoxins are of great concern because of their detrimental effects on the health of humans and animals, including carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (3). Aflatoxins are also of industrial importance due to the economic losses resulting from condemnation of contaminated crops, cheese defects, and impaired growth and feed efficiency of animals fed contaminated feeds. Consequently there is a great demand for novel strategies to prevent both the formation of aflatoxin in foods and feeds and the impact of existing aflatoxin contamination.

Previous studies have shown that two probiotic strains, *Lactobacillus rhamnosus* strain GG (ATCC 53103) and *L. rhamnosus* strain LC-705 (DSM 7061), efficiently remove AFB_1 from solution (4–7). Theoretical calculations by Oatley et al. (18) demonstrate that AFB_1 removal does not arise solely from trapping of the toxin in the bacterial pellet during centrifugation. Metabolic conversion and covalent binding of AFB_1 by the bacteria have been excluded as a mechanism of removal, and noncovalent binding of AFB_1 to the bacteria has been proposed. These strains reduce tissue uptake of AFB_1 from the duodenum of chicks (8) and may permit detoxification of the human diet through reducing aflatoxin absorption in the gastrointestinal tract. It is important that nonviable bacteria also have high binding ability, as survival of viable bacteria is reduced upon passing through the stomach at low pH. Similar mutagen-binding abilities have been reported for viable and nonviable (heat-treated) bacteria (19, 26, 27). Nonviable (heat- and acid-treated) strains GG and LC-705 bind AFB_1 as effectively as viable bacteria (7).

An understanding of the nature of the binding, for example, if binding is intracellular or extracellular or reversible or irreversible, is important in understanding the fate of bound aflatoxin. If aflatoxin is bound noncovalently and extracellularly, it may be released by the continual washing of the bacterial surface in the gastrointestinal tract if the binding is insufficiently strong. Potential future applications of this method to reduce aflatoxin bioavailability in animals or humans rely on the relative stability of the complex formed. This work was designed to clarify whether binding occurs extracellularly and to assess the stability of the complexes formed.

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MATERIALS AND METHODS

Bacteria. All strains were cultured for 24 h in deMan-Rogosa-Sharpe (MRS) broth (Oxoid, Hampshire, United Kingdom [UK]) under aerobic conditions (37°C, 5% CO_2) except for *Escherichia coli*, which was grown in nutrient broth (Oxoid), and *Propionibacterium freudenreichii* subsp. *shermanii* JS, which was grown under anaerobic conditions in yeast extract-sodium lactate (YEL) broth (prepared according to Malik et al. [16]). *L. rhamnosus* strain GG (ATCC 53103), *L. rhamnosus* strain LC-705 (DSM 7061) and *P. freudenreichii* subsp. *shermanii* JS were lyophilized powders supplied by Valio Ltd., Helsinki, Finland. *L. delbrueckii* subsp. *bulgaricus, Lactococcus lactis* subsp. *cremoris, L. helveticus, Lactococcus lactis* subsp. *lactis, Streptococcus thermophilus*, and *L. plantarum* (ATCC 8014) were from the Australian Starter Culture Research Centre Collection, Werribee, Australia. *L. acidophilus* (ATCC 4356) and *E. coli* were from the Department of Medical Laboratory Science Collection, RMIT-University, Melbourne, Australia. *L. casei* Shirota (YIT 901) and *L. acidophilus* strain LC1 were isolated from commercial products. These strains were selected based either on their common use by food industry or on available information regarding their effects on food mutagens.

Bacterial counts were determined by flow cytometry using a Coulter Electronics EPICS Elite ESP cytometer equipped with an air-cooled 488-nm argon-ion laser at 15 mV. For the complex stability experiments, viability of the 24-h cultures was assessed using the fluorescent emission from Sytox green nucleic acid stain (L-7020; Molecular Probes, Eugene, Oreg.) at 1 μM per 10^6 to 10^7 bacteria and found to be 95 to 99% for each strain. In the surface binding experiments, total bacterial counts were enumerated using Syto9 (Live/Dead *Bac*Light bacterial viability kit L-7012; Molecular Probes) at 3.34 μM per 10^6 to 10^7 bacteria. A 525-nm bandpass filter was used to collect the emission for both stains, and Fluoresbrite beads (2.0 μm; Polysciences Inc.) were used as an internal calibration.

Cultured bacteria were washed twice (4 ml of phosphate-buffered saline [PBS; pH 7.3, 0.01 M]) prior to use. In the complex stability experiments, cultured bacterial samples (10^{10} bacteria) were used. In the surface binding experiments, both cultured (10^9 bacteria) and lyophilized (10^{10} bacteria) bacterial samples were used. Bacteria were either incubated as viable (in 4 ml of PBS for 1 h), heat treated (boiled in 4 ml of PBS for 1 h), or acid treated (incubated in 4 ml of 2 M HCl for 1 h). Acid-treated bacteria were then washed twice (4 ml of PBS). All bacterial samples were centrifuged, and the supernatant was removed prior to AFB_1 binding assays. All incubations were carried out at 37°C, and all centrifugations were at 2,500 × *g* for 10 min (<10°C) unless indicated otherwise.

The structural integrity of the bacterial cell walls was tested using light microscopy and a Gram stain (20).

Aflatoxin binding assay. AFB_1 has been classified as a class I human carcinogen (14). AFB_1 (Sigma, St. Louis, Mo.) was dissolved in benzene-acetonitrile (97:3, vol/vol), and the concentration was determined spectrophotometrically at 348 nm (ε_{348} = 19,800 M^{-1} cm^{-1}). To prepare an aqueous solution, either benzene-acetonitrile was evaporated with nitrogen and 50 μl of methanol added and then made to volume with PBS, or PBS was added directly and the benzene-acetonitrile was evaporated by heating in a waterbath at 80°C for 10 min.

The bacterial pellet was suspended in PBS (1.5 ml) containing either 5 μg (complex stability experiments) or 10 ng of AFB_1 (surface binding experiments) per ml, incubated at 37°C for either 4 h (complex stability experiments) or 30 min (surface binding experiments), and centrifuged prior to analysis by either high performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA). All assays were performed in duplicate, and both positive controls (PBS substituted for bacteria) and negative controls (PBS substituted for AFB_1) were included.

HPLC. Reverse-phase HPLC was used to quantify AFB_1 remaining in the supernatant of bacteria incubated with AFB_1. The HPLC system (Applied Biosystems) was fitted with a dual-pump model 400 solvent delivery system, a model 980 programmable fluorescence detector, and an ODS Spheri-5 Brownlee column (220 mm by 4.6 mm, 5 μm; Perkin Elmer) fitted with a C_{18} guard column (Perkin Elmer). Water-acetonitrile-methanol (60:30:10, vol/vol/vol) was used as the mobile phase, with a flow rate of 1 ml/min. The assay was carried out at room temperature with an injection volume of 50 μl. Detection was done by fluorescence with excitation and emission wavelengths of 365 and 418 nm, respectively. The retention time was 10 min. Chromatograms were recorded at a chart speed of 0.5 cm/min and a peak width of 0.4 min. The percentage of AFB_1 removed was calculated using the equation 100 × [1 − (peak area of AFB_1 in the supernatant)/(peak area of AFB_1 in the positive control)].

Complex stability. The stabilities of the bacteria-AFB_1 complexes were evaluated by determining the amount of AFB_1 remaining bound following five washes. Bacterial pellets were washed by being suspended in Milli-Q water (1.5 ml) and incubated at room temperature for 10 min. After centrifugation, the supernatant was removed, released AFB_1 was quantified by HPLC, and the percent AFB_1 bound was calculated. This washing procedure was repeated another four times. In addition, the stability of AFB_1 bound to viable and nonviable *L. rhamnosus* strains after five aqueous washes was tested by autoclaving in PBS (121°C, 40 min), sonicating in PBS (40 min in an ice-water bath [<10°C]; Soniclean ultrasonic cleaner; Transtek Systems), and by suspending the washed pellet in chloroform (5 ml).

The stabilities of the viable *L. rhamnosus*-AFB_1 complexes were also evaluated under various conditions; pH (2, 7 and 10), temperature (4, 25, and 37°C), and a series of solvents (methanol, acetonitrile, chloroform, and benzene). Following AFB_1 binding, the bacterial pellet was suspended in 5 ml of either HCl (pH 2), water (pH 7), NaOH (pH 10), methanol, acetonitrile, chloroform, or benzene and incubated for 1 h at either 4, 25, or 37°C. Solvent incubations were only carried out at 37°C. Following centrifugation, chloroform (2 ml) was added to the supernatant (2 ml), the layers were separated, chloroform was evaporated, and the residue was dissolved in methanol (1 ml) for HPLC analysis of AFB_1. For the methanolic supernatant, 2 ml was reduced to 1 ml by evaporation, and for the chloroform supernatant, 2 ml was evaporated to dryness and the residue was reconstituted in methanol (1 ml).

ELISA. Microtiter plate wells (Grenier Labrotechnik), were coated with 50 μl of bovine serum albumin (BSA)-AFB_1 in PBS (0.1 μg/ml) (Sigma), dried at 35°C for over 20 h, and stored at 4°C. Prior to use, plates were washed five times with 0.05% Tween 20 (Sigma) in PBS (PBS-Tween 20), the first time by full immersion (3 min) and the remaining washes with a multichannel pipette. Nonspecific binding sites were blocked with 150 μl of 0.35% gelatin (Sigma) in PBS (PBS-gelatin) for 60 min at room temperature and washed twice with PBS-Tween 20 before use. Bacteria were incubated with AFB_1 (10 ng/ml of PBS) for 30 min at 37°C or with PBS for bacterial controls. After centrifugation, the pellet was washed twice with PBS to remove any loosely bound AFB_1. Supernatant, both washings, and the pellet were retained for the competitive inhibition assay. Competitive binding was carried out by adding 200 μl of rabbit polyclonal antibody (anti-AFB_1), diluted 1:2,500 in PBS-gelatin, to tubes containing 200 μl of either standard AFB_1, bacterial supernatant, or bacterial washing or by suspending the bacterial pellet in an equal volume of anti-AFB_1 to maintain a twofold dilution. Where necessary, PBS was added to the pellets to make the volume up to 200 μl in order to have sufficient sample solution for the plate wells. Tubes were vortexed and incubated at 37°C for 60 min. These mixtures (50 μl, six replicates) were dispensed into the wells of washed microtiter plates and incubated at 37°C for 90 min. After washing five times with PBS-Tween 20, binding was assayed by adding 50 μl of 1:30,000 goat anti-rabbit immunoglobulin G-peroxidase (Sigma) in PBS to each well and incubating the plate for 90 min at 37°C. Plates were washed five times with PBS-Tween 20 and once with Milli-Q water.

Substrate solution was prepared by adding 300 μl of 3,3′,5,5′-tetramethylbenzidine (Sigma) (3.6 mg in 360 μl of dimethyl sulfoxide) to 30 ml of sodium acetate buffer (0.1 M, pH 6.0) following incubation of the two separate solutions for 30 min at 37°C. After incubation of the mixture for another 30 min and just prior to addition to the plate, 6 μl of hydrogen peroxide (prepared by addition of 10 μl of 30% [wt/vol] hydrogen peroxide [BDH] to 90 μl of Milli-Q water) was added to the substrate solution. Bound peroxidase was detected by the addition of 50 μl of substrate solution to the wells. Following a 30-min incubation at room temperature, the reaction was stopped by addition of 50 μl of 2 M H_2SO_4. The optical density at 450 nm (OD_{450}) was read on a DIAS plate reader (Dynatech, Guernsey, UK). The inhibition of antibody (anti-AFB_1) binding to immobilized antigen (AFB_1-BSA) was calculated by using the following equation: % inhibition = 100 × (1 − [OD^s/OD^c]), where OD^s is the mean absorbance of the supernatant or bacterial pellet and OD^c is the mean absorbance of the negative control (PBS).

Statistical analysis. Significant differences between ELISA samples, bacterial strains, and bacterial treatments were tested by analysis of variance using Minitab. Data were normalized, and Tukey tests were performed. The results of the complex stability experiments were subjected to Student's *t* test to identify significant differences between bacterial strains and bacterial treatments. Probability (*P*) values of <0.05 were considered significant.

RESULTS

Complex stability. None of the strains tested (Table 1) were more efficient in binding AFB_1 than the two reported previously, GG and LC-705. The complexes formed between AFB_1

TABLE 1. Percentage of AFB_1 bound on exposure to viable and heat- and acid-treated bacteria and remaining bound after up to five washes with Milli-Q water[a]

Strain	% AFB_1 bound[b] ± SD					
	Viable		Heat treated		Acid treated	
	Initial	Final	Initial	Final	Initial	Final
L. rhamnosus GG	78.9 ± 1.9	49.5 ± 0.1	84.1 ± 2.3	66.4 ± 1.7	86.7 ± 0.4	71.3 ± 0.3
L. rhamnosus LC-705	76.5 ± 3.7	37.9 ± 8.1	87.8 ± 0.6	70.5 ± 0.2	88.3 ± 3.5	71.1 ± 4.06
L. acidophilus LC1	59.7 ± 6.4	21.2 ± 0.6	74.7 ± 0.3	34.8 ± 1.5	84.2 ± 0.2	56.6 ± 6.5
L. lactis subsp. *lactis*	59.0 ± 12.6	24.8 ± 9.3	58.1 ± 0.9	25.6 ± 2.7	69.5 ± 6.0	31.7 ± 2.9
L. acidophilus ATCC 4356	48.3 ± 2.0	13.5 ± 1.0	69.7 ± 2.1	35.5 ± 4.8	81.3 ± 6.3	52.6 ± 6.5
L. plantarum	29.9 ± 2.9	0.6 ± 1.7 (2)	35.5 ± 1.1	9.1 ± 4.7	62.7 ± 0.8	16.0 ± 2.3
L. casei Shirota	21.8 ± 0.8	0.5 ± 2.9 (3)	41.5 ± 2.4	10.0 ± 1.7	32.3 ± 1.0	1.7 ± 0.5 (3)
L. delbrueckii subsp. *bulgaricus*	15.6 ± 2.1	1.9 ± 3.9 (1)	33.7 ± 3.1	2.5 ± 1.9 (3)	75.8 ± 1.1	35.8 ± 1.8
L. helveticus	17.5 ± 3.7	6.0 ± 0.2 (2)	29.8 ± 3.3	6.0 ± 0.2 (3)	58.1 ± 2.9	26.5 ± 3.3
P. freudenreichii subsp. *shermanii* JS	22.3 ± 1.7	2.4 ± 3.9 (2)	67.3 ± 2.3	29.6 ± 1.7	82.5 ± 7.9	53.9 ± 2.0
Lactococcus lactis subsp. *cremoris*	26.9 ± 4.3	3.8 ± 1.4 (1)	40.1 ± 5.5	1.6 ± 1.9 (3)	43.7 ± 2.9	4.9 ± 3.3 (3)
Streptococcus thermophilus	32.7 ± 4.3	19.8 ± 4.5 (3)	42.0 ± 0.7	18.7 ± 1.9 (4)	63.8 ± 0.3	30.4 ± 5.2

[a] Bacteria were incubated in PBS (4 ml) at 37°C for 1 h (viable), boiled in PBS (4 ml) for 1 h (heat treated), or incubated in 2 M HCl (4 ml) at 37°C for 1 h (acid treated).

[b] Initial, percentage of AFB_1 removed after 10^{10} bacteria were incubated with AFB_1. Final, percentage of AFB_1 remaining bound to the bacteria after five washes (1.5 ml each) with Milli-Q water unless otherwise stated. When the amount of AFB_1 released was below the HPLC limit of detection prior to the fifth wash, AFB_1 remaining bound after the first, second, third, or fourth wash was used, as indicated in parentheses. Results are the average ± SD for duplicate samples.

and GG or LC-705 were also significantly more stable than those formed with the other strains tested ($P < 0.05$) (Table 1), and these strains were selected for further study (Tables 2 and 3). Heat and acid treatments have a significant impact on both the amount of AFB_1 bound and its retention for most of the strains tested (Table 1). After washing, viable LC-705 retained 38% of the AFB_1 initially bound (Table 1). This was significantly less than the 50% retained for viable GG and the 66 to 71% retained for heat or acid treatment of either strain ($P < 0.05$). After five aqueous washes, further autoclaving and sonication did not release any detectable AFB_1 from viable or nonviable GG or LC-705 pellets (Table 2). Between 87 and 96% of the AFB_1 remaining bound after five aqueous washes was recovered by extraction with chloroform.

A wider range of extraction solutions were used for studying the stability of viable GG-AFB_1 and LC-705-AFB_1 complexes. Only 6 to 11% of bound AFB_1 was released from these complexes in water at pH 2, 7, and 10 and temperatures of 4, 25, and 37°C (Table 3). Suspending the complex in methanol, acetonitrile, chloroform, or benzene at 37°C released 83 to 99% of bound AFB_1 (Table 3), with chloroform being the most effective.

Structural integrity. Methanol, acetonitrile, chloroform, benzene, and boiling did not appear to affect the structural integrity, shape, and size of either GG or LC-705. Autoclaving and sonication appeared to decrease rod length. Sodium hydroxide (1 M) appeared to have an effect on rod length and a slight effect on structural integrity. Hydrochloric acid disrupted structural integrity, with a greater effect seen at 2 M than at 1 M concentration.

Surface binding. Results obtained by ELISA for cultured (Table 4) and lyophilized (Table 5) bacteria indicated that the majority of bound AFB_1 is attached to the bacterial surface in an orientation recognized by the highly specific anti-AFB_1. Significantly more AFB_1 was detected on the cultured pellets and in the first wash of GG, LC-705, and *L. casei* Shirota than on *E. coli* ($P < 0.05$). For GG, LC-705, and *L. casei* Shirota, there were significant differences between the amounts of AFB_1 detected in each sample type ($P < 0.05$), but no significant differences between the strains. For the lyophilized bacteria, there was no significant difference between the strains or treatments. In contrast to the cultured strains, (Table 4), washing of the lyophilized bacteria released large amounts of AFB_1 (Table 5). There was significantly more AFB_1 in the washings than on the pellet for acid-treated GG and in the supernatant and first wash compared to the pellet for acid-treated LC-705 ($P < 0.05$). For viable LC-705, the amount of AFB_1 on the pellet was significantly less than in all other samples ($P < 0.05$).

TABLE 2. Effect of autoclaving (121°C, 40 min), sonication (<10°C, 40 min), and chloroform extraction (5 ml) on release of AFB_1 remaining bound to viable and heat- or acid-treated *L. rhamnosus* strains GG and LC-705 following five washes with Milli-Q water[a]

Treatment	% Bound AFB_1 released ± SD					
	L. rhamnosus GG			*L. rhamnosus* LC-705		
	Viable	Heat treated	Acid treated	Viable	Heat treated	Acid treated
Autoclaving	ND	ND	ND	ND	ND	ND
Sonication	ND	ND	ND	ND	ND	ND
Chloroform extraction	97.3 ± 2.3	87.2 ± 4.1	91.8 ± 2.8	96.1 ± 4.0	89.3 ± 3.1	93.2 ± 2.9

[a] Bacteria were initially incubated with AFB_1 and then washed five times (1.5 ml each with Milli-Q water. Results are the average ± SD for duplicate samples. Also see Table 1, footnote *a*.

[b] ND, not detected; the amount released was below the detection limit (0.01 μg/ml) of the HPLC method used.

TABLE 3. Percentage of AFB_1 bound to *L. rhamnosus* strain GG or LC-705 released on incubation at a range of temperatures with solutions (5 ml) of different pHs and a range of solvents[a]

Treatment	% Bound AFB_1 released ± SD					
	L. rhamnosus GG			*L. rhamnosus* LC-705		
	4°C	25°C	37°C	4°C	25°C	37°C
HCl (pH 2)	8 ± 3	8 ± 3	6 ± 1	7 ± 1	10 ± 5	8 ± 2
Water (pH 7)	7 ± 1	6.4 ± 0.6	6.1 ± 0.5	7 ± 4	10 ± 1	8 ± 1
NaOH (pH 10)	9 ± 4	8 ± 4	7 ± 2	9 ± 1	11 ± 1	8 ± 2
Methanol			83 ± 5			88 ± 3
Acetonitrile			87 ± 4			90 ± 5
Chloroform			99 ± 2			98.3 ± 0.6
Benzene			89 ± 3			87 ± 4

[a] Initial binding was achieved by incubating bacteria with 1.5 ml of AFB_1 (5 μg/ml) in PBS for 1 h at 37°C. Results are the average ± SD for duplicate samples.

TABLE 5. AFB_1 detected by anti-AFB_1 antibody in bacterial supernatants (1.5 ml), bacterial washes (4 ml each), and bacterial pellets (10^{10} bacteria) of lyophilized *L. rhamnosus* GG and LC-705[a]

Strain[b]	AFB_1 (ng) ± SD			
	Supernatant	First wash	Second wash	Pellet
GG				
Viable	3.43 ± 0.00	5 ± 2	2.8 ± 0.1	2.4 ± 0.7
Heat treated	2.7 ± 0.4	3.4 ± 0.4	6.9 ± 0.9	3 ± 1
Acid treated	4.3 ± 0.9	18 ± 18	6 ± 2	0.8 ± 0.5
LC-705				
Viable	9 ± 1	5.7 ± 0.5	4.8 ± 0.7	0.47 ± 0.06
Heat treated	6 ± 1	4 ± 1	5 ± 2	1.5 ± 0.3
Acid treated	7.34 ± 0.00	23 ± 20	5 ± 1	1.2 ± 0.7

[a] Bacteria were incubated with 1.5 ml of AFB_1 (10 ng/ml) in PBS for 30 min at 37°C. Results are the average ± SD for duplicate samples.
[b] See Table 1, footnote *a*.

DISCUSSION

Complex stability. In previous studies of the binding of mutagens to LAB, both reversible and irreversible binding has been reported (17, 19). Release of bound mutagens can be very specific to the washing solution used. For example, bacterial bound 3-amino-1,4-dimethyl-5H-pyrido(4,3-*b*)indole (Trp-P-1) is effectively extracted by aqueous methanol, ethanol, alkalized ethylacetate, ammonia, and solutions of $MgCl_2$ and $CaCl_2$, but little is extracted by water and solutions of KCl, NaCl, and buffers at various pHs (28, 29). All strains tested here show reversible binding of AFB_1 when washed with water (Table 1). There is considerable variation in the percentage of AFB_1 bound both initially and after up to five washes. Of the strains tested here, GG and LC-705 were most effective in initially binding and also retaining AFB_1, suggesting that the complexes formed with these strains were the most stable.

Treatment of bacteria with heat or acid may affect the AFB_1 binding mechanism. Viable *Flavobacterium aurantiacum* removes AFB_1 irreversibly from solution by metabolic degradation following the formation of a loose complex with the periphery of the bacteria (1, 15, 23). In this case no AFB_1 is recovered by washing with water, sonication, and subsequent washing of the ruptured bacteria with water, or extraction of either intact or ruptured bacteria with chloroform. However, heat-treated *F. aurantiacum* binds AFB_1 reversibly, releasing AFB_1 when washed with water (1, 15). Heat and acid treatment significantly enhanced the stability of the complex formed with AFB_1 for a number of strains, including GG and LC-705, and no significant change in stability was observed for the remaining strains (Table 1). The stability of complexes formed between AFB_1 and heat-treated *Bifidobacterium* strains is reported to be similar to that of heat-treated GG (18). Heat and acid treatments also significantly enhanced the ability of bacteria to remove AFB_1, with acid treatment being more effective than heat treatment in most cases. However, other studies (9) have shown that the relative amounts of AFB_1 removed by viable and heat- and acid-treated bacteria depend on initial AFB_1 concentrations.

Cell wall polysaccharide and peptidoglycan (17, 21, 25, 28) are the two main elements responsible for the binding of mutagens to LAB (13, 28, 30). Both of these components are expected to be greatly affected by heat and acid treatments. Heat may cause protein denaturation or the formation of maillard reaction products between polysaccharides and peptides or proteins. Acid may break the glycosidic linkages in polysaccharides, releasing monomers that may then be further fragmented into aldehydes. Acid may also break the amide linkages in peptides or proteins to produce peptides and the component amino acids. Hence, acid treatment may break down the peptidoglycan structure, resulting in the observed compromised structural integrity. Although the peptidoglycan layer is quite thick in these organisms, there may be a decrease in thickness, reduction in cross-links, and/or increase in pore size. This perturbation of the bacterial cell wall may allow AFB_1 to bind to cell wall and plasma membrane constituents that are not available when the bacterial cell is intact. The effective removal of AFB_1 by all nonviable bacteria suggests that binding rather than metabolism is involved in all cases.

Autoclaving and sonication did not release any detectable AFB_1 from bacterial pellets that had been washed five times with water (Table 2). Autoclaving causes denaturation of bacterial proteins and enzymes, whereas sonication releases molecules loosely bound to the surface of the bacteria. Due to the thick peptidoglycan of these *L. rhamnosus* strains, the bacteria are not lysed by this sonication. These results suggest that denaturation by high temperatures does not cause the most strongly bound AFB_1 to be released and that this AFB_1 is not bound to loosely attached bacterial components.

TABLE 4. AFB_1 detected by anti-AFB_1 antibody in bacterial supernatants (1.5 ml), bacterial washes (4 ml each), and bacterial pellets (10^9 bacteria) of 24-h cultures of *L. rhamnosus* GG, *L. rhamnosus* LC-705, *L. casei* Shirota, and *E. coli*

Strain	AFB_1 (ng)[a] ± SD			
	Supernatant	First wash	Second wash	Pellet
L. rhamnosus GG	8.1 ± 0.8	3.7 ± 0.1	ND[b]	0.14 ± 0.03
L. rhamnosus LC-705	8.68 ± 0.00	3.0 ± 0.2	ND	0.12 ± 0.03
L. casei Shirota	12 ± 4	2.39 ± 0.06	ND	0.10 ± 0.00
E. coli	13 ± 3	ND	ND	ND

[a] Bacteria were incubated with 1.5 ml of AFB_1 (10 ng/ml) in PBS for 30 min at 37°C. Results are the average ± SD for duplicate samples.
[b] ND, not detected; below the detection limit of 0.3 ng/ml.

Morotomi and Mutai (17) suggested that release of a heterocyclic amine in alkaline but not neutral solution was due to a cation-exchange mechanism. Only around 10% of bound AFB_1 is released by aqueous solutions of pH 2 to 10 (Table 3), representative of the gastrointestinal tract pH range. Binding of AFB_1 to GG has been reported to be unaffected by pH in the range from 2.5 to 8.5 (10), suggesting that a cation-exchange mechanism is not operating, as supported by the data in Table 3.

Optimal temperatures for initial removal of AFB_1 from solution by *Flavobacterium aurantiacum*, GG, and LC-705 have been reported to be 35°C (15), 37°C, and 37°C (6), respectively. However, incubation temperature did not significantly affect the stability of the complexes formed between either GG or LC-705 and AFB_1 in the range from 4 to 37°C (Table 3).

Organic solvents released almost all AFB_1 bound to GG and LC-705, providing further evidence of noncovalent binding. The order of effectiveness of extraction, methanol < acetonitrile = benzene < chloroform, does not match the order of decreasing polarity. This may be because the hydrophobicity of the AFB_1 molecule most closely matches that of chloroform. Hydrophobic interactions play a major role in the binding mechanism (10). The Gram stain results suggest that chloroform extraction does not expose the intracellular lipophilic membrane, but rather only extracts extracellular components.

Surface binding. To determine the location of AFB_1 binding, an indirect competitive inhibition method of ELISA was used as a measure of bacterial surface binding. The principle of this assay is based on the polyclonal antibody raised against AFB_1 (anti-AFB_1) binding AFB_1 that is either bound to the bacterial surface or remaining free in the bacterial supernatant and thus reducing the amount of anti-AFB_1 available to bind the AFB_1-BSA coating the microtiter plate wells. The reduction in absorbance, by comparison with the negative control (PBS), is a measure of the amount of AFB_1 in the sample that is accessible to anti-AFB_1.

It has previously been concluded that bacterial cells should exclude molecules larger than 55 kDa (2), and hence anti-AFB_1 should not penetrate the cell wall. To our knowledge, this is the first time that ELISA results have been used as evidence of surface binding. Strains other than those showing significant efficiency in removing AFB_1 were included for comparison. Percentages of AFB_1 bound to GG, LC-705, *L. casei* Shirota, and *E. coli* are reported to be 78, 79, 33, and 16%, respectively (6). The relative amounts of AFB_1 detected on the pellets (Table 4) correlate well with this order. For cultured GG, LC-705, and *L. casei* Shirota, a gradual decrease in AFB_1 is observed between the supernatant, first wash, and second wash and then an increase in AFB_1 for the pellet, as expected.

The structural integrity of acid-treated bacteria appears to be compromised, and this may permit binding of AFB_1 to intracellular components. The lower level of AFB_1 detected on the acid-treated GG pellets compared to the heat-treated pellets may be because the size of the antibody molecule inhibits its ability to pass through the disrupted cell wall and bind to intracellular AFB_1.

The variation between cultured and lyophilized bacterial pellets may arise from different concentrations of bacteria, as this has previously been shown to be significant (6). Culturing may also induce changes in the cell wall components, as the medium used for culturing may differ from that used for the lyophilized bacteria. In some cases, not all AFB_1 is accounted for on the surface of the bacteria. This may be due to the binding of some AFB_1 on the surface in an orientation that cannot be recognized by the antibody, or alternatively, a small amount of AFB_1 may penetrate the cell wall. Exposure of these bacteria to enzymes does not completely destroy the AFB_1 binding site (10). This may be due to some nonsurface binding or blocking of the enzymes. The high errors inherent in the ELISA method prevent accurate AFB_1 accounting. The greater percentage of bound AFB_1 released in the ELISA experiments (Tables 4 and 5) relative to the complex stability experiments (Tables 1 to 3) may be due to the larger washing volume used and different initial concentrations of AFB_1.

Conclusions. The reversibility of binding was demonstrated by the effect of bacterial washing. This suggests that AFB_1 is bound to the bacteria by weak noncovalent interactions, such as associating with hydrophobic pockets on the bacterial surface. Results from ELISA, light microscopy, and solvent extractions are consistent with AFB_1 binding predominantly to the extracellular bacterial surface of both GG and LC-705, whether viable or heat treated. The detection of AFB_1 by ELISA suggests that most of the AFB_1 is bound to bacterial surfaces; however, these surfaces may be intracellular in the case of acid-treated bacteria rather than extracellular. The recovery of almost all bacterially bound AFB_1 by washing with chloroform is further evidence of surface binding. Present knowledge on the surface characteristics of GG and LC-705 is very limited; however, these strains do not possess an S-layer but rather are encompassed by a polysaccharide capsule during growth and have a surface which is hydrophilic in nature. Since the bacterial surface will change during growth, investigations into the effect of growth phase on AFB_1 binding are currently under way.

This study shows that only small amounts of bound AFB_1 are released from the bacterial surface in aqueous solution between pH 2 and 10 at human body temperature. However, full in vivo studies are required to assess the effects of these bacteria on the bioavailability and mutagenicity of consumed aflatoxins. Bacterial removal of aflatoxins from food and feed prior to their consumption may be a more effective decontamination method, but this will involve an additional processing step.

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