# *Lactobacillus rhamnosus* Strain GG Reduces Aflatoxin $B_1$ Transport, Metabolism, and Toxicity in Caco-2 Cells[▽]

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The probiotic *Lactobacillus rhamnosus* GG is able to bind the potent hepatocarcinogen aflatoxin $B_1$ ($AFB_1$) and thus potentially restrict its rapid absorption from the intestine. In this study we investigated the potential of GG to reduce $AFB_1$ availability in vitro in Caco-2 cells adapted to express cytochrome P-450 (CYP) 3A4, such that both transport and toxicity could be assessed. Caco-2 cells were grown as confluent monolayers on transmembrane filters for 21 days prior to all studies. $AFB_1$ levels in culture medium were measured by high-performance liquid chromatography. In CYP 3A4-induced monolayers, $AFB_1$ transport from the apical to the basolateral chamber was reduced from 11.1% ± 1.9% to 6.4% ± 2.5% ($P = 0.019$) and to 3.3% ± 1.8% ($P = 0.002$) within the first hour in monolayers coincubated with GG ($1 \times 10^{10}$ and $5 \times 10^{10}$ CFU/ml, respectively). GG ($1 \times 10^{10}$ and $5 \times 10^{10}$ CFU/ml) bound 40.1% ± 8.3% and 61.0% ± 6.0% of added $AFB_1$ after 1 h, respectively. $AFB_1$ caused significant reductions of 30.1% ($P = 0.01$), 49.4% ($P = 0.004$), and 64.4% ($P < 0.001$) in transepithelial resistance after 24, 48, and 72 h, respectively. Coincubation with $1 \times 10^{10}$ CFU/ml GG after 24 h protected against $AFB_1$-induced reductions in transepithelial resistance at both 24 h ($P = 0.002$) and 48 h ($P = 0.04$). DNA fragmentation was apparent in cells treated only with $AFB_1$ cells but not in cells coincubated with either $1 \times 10^{10}$ or $5 \times 10^{10}$ CFU/ml GG. GG reduced $AFB_1$ uptake and protected against both membrane and DNA damage in the Caco-2 model. These data are suggestive of a beneficial role of GG against dietary exposure to aflatoxin.

Mycotoxins are fungal metabolites contaminating 25% of cereals and grains destined for human consumption (3). One of the most toxic groups of mycotoxins are the aflatoxins (20, 47), and aflatoxin $B_1$ ($AFB_1$), the most toxic and carcinogenic of the naturally occurring aflatoxins, has been classified as a class 1A human carcinogen by the International Agency for Research on Cancer (20). Aflatoxins additionally cause growth impairment and immune suppression in a range of animal species (48). In West Africa, dietary aflatoxin exposure is frequent and occurs at high levels (12, 13, 38–42) and has been associated with growth faltering (12, 13, 39) and immune suppression (39) in exposed children. Aflatoxins are efficiently absorbed in the intestinal tract, probably by passive diffusion (19, 32). $AFB_1$ is subsequently metabolized predominantly by cytochrome P-450 (CYP) enzyme systems (16) to produce a range of metabolites, including $AFM_1$ (by CYP 1A2) and $AFQ_1$ (by CYP 3A4). Aflatoxicol (AFL), regarded as a reservoir for $AFB_1$, can also be generated by cytosolic reductases (5). However, the toxicity of $AFB_1$ is generally regarded to occur via production of the highly reactive $AFB_1$-8,9-epoxide (predominantly via CYP 3A4 but also by CYP 1A2), which forms covalent adducts with macromolecules such as proteins and DNA (5). This reactive epoxide is capable of causing damage to cells in the liver and at the intestinal interface. Given the observations of an association between aflatoxin

exposure and growth faltering in West African infants (12, 13), it is possible that direct damage within the intestine may be occurring that alters nutrient uptake or leads to the "intestinal leakiness" that has been associated with growth faltering in Gambian infants by Lunn (25) at the time when weaning foods are introduced. Such weaning foods are commonly less hygienic than breast milk and additionally are likely to be contaminated with aflatoxins at high levels (12, 13). Considering the multiple adverse effects of aflatoxin exposure, the reduction of intestinal uptake and transport to the body are of major interest to reduce or prevent toxic events.

Intervention approaches at the individual level use either sorbent materials to prevent aflatoxin absorption in the gastrointestinal tract (enterosorption) or compounds that alter activation or detoxification of $AFB_1$ (chemoprotection) (48). There is considerable interest in the protective role of probiotic bacteria in humans (33). Our previous work on >250 strains of lactic acid bacteria isolated from either dairy products or healthy human microbiota revealed that the efficacy of aflatoxin binding was highly variable depending on the genus and strain of bacteria. Two *Lactobacillus rhamnosus* strains, GG and LC-705, were the most efficient strains in binding a range of mycotoxins, including aflatoxins (6, 9, 10, 18, 28, 29). Carbohydrate and protein components of the bacterial surface are important for $AFB_1$ binding (17, 18), and heat treatment does not reduce this binding (6, 23). *Lactobacillus rhamnosus* GG is currently used in various dairy products including yogurt and is therefore a good candidate for assessing protective effects with a view to its use in humans. Additionally, evidence from animal studies showed that $AFB_1$ binding by probiotic

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bacteria successfully reduces tissue uptake of $AFB_1$ in the duodenum of chicks (8, 14).

The aim of this study was to investigate the modulation of $AFB_1$ transport by GG in the human intestinal cell line Caco-2. This cell line has previously been used as an in vitro model for studying the intestinal absorption of xenobiotics, including mycotoxins (1, 26). In this study, we used Caco-2 cells as a confluent monolayer and allowed differentiation to occur for 21 days to develop an apical brush border and to allow tight junction formation between cells (34). Further, we utilized the Caco-2 cell model to investigate the modulation of $AFB_1$ metabolism and intestinal cell toxicity in the presence of GG. Since the expression of aflatoxin-metabolizing enzymes in the Caco-2 cells differs from that seen for normal human enterocytes, induction of one of the key activating enzymes (CYP 3A4) was established by preincubation with $1\alpha,25$-dihydroxy vitamin $D_3$ [$1,25(OH)_2D_3$] (35).

## MATERIALS AND METHODS

**Bacterial strains.** *Lactobacillus rhamnosus* strain GG (ATCC 53013) was obtained from Valio Ltd. (Helsinki, Finland) as lyophilized powder and stored at $-80°C$. Bacteria were weighed, suspended in phosphate-buffered saline, boiled for 1 h in a water bath, and centrifuged ($3,000 \times g$ for 7 min, $4°C$). After being washed once with culture medium (Dulbecco's modified Eagle's medium [DMEM]; Cambrex Bio Science, Verviers, Belgium) containing antibiotics/antimycotics (0.2% penicillin, streptomycin, amphotericin B; Invitrogen, Paisley, United Kingdom), bacteria were centrifuged again and then suspended in DMEM containing antibiotics, resulting in final concentrations of $1 \times 10^{10}$ or $5 \times 10^{10}$ CFU/ml.

**Cell line and culture conditions.** The human colon cancer cell line Caco-2 (ATCC HTB-37) was cultivated according to standard procedures in DMEM with 20% fetal bovine serum (Invitrogen, Paisley, United Kingdom) and 0.2% antibiotic/antimycotic, and cultures were kept at $37°C$ at 5% $CO_2$. Cells were grown in 12-well plates until confluent monolayers formed and cells differentiated into small intestinal cells containing microvilli in 21 days.

**Cytochrome 3A4 induction with $1,25(OH)_2D_3$.** The Caco-2 cell line has a low expression of the key enzyme CYP 3A4, which generates the aflatoxin epoxide. This enzyme was induced by adding 0.25 $\mu$M $1,25(OH)_2D_3$ in DMEM (35) on day 21 of culture and incubating for 2 or 5 days prior to the addition of $AFB_1$ and GG in culture medium. In all experiments, the bacterial suspension was applied to Caco-2 cells first and immediately followed by $AFB_1$ solution in DMEM prior to further incubation.

**Uptake of $AFB_1$ and formation of free metabolites.** For transport experiments, cells were grown on Transwell filter inserts (12-mm diameter, 0.4-$\mu$m pore size; Corning Inc., Corning, NY) with medium volumes of 1 ml in the apical and 2 ml in the basolateral chamber. Medium was changed three times per week, and cells were differentiated for 21 days. Following induction with $1,25(OH)_2D_3$ for 2 days, the transport of $AFB_1$ from the apical to the basolateral side was investigated. Either $AFB_1$ (150 $\mu$M) alone in culture medium or GG suspension ($1 \times 10^{10}$ or $5 \times 10^{10}$ CFU/ml) and $AFB_1$ (150 $\mu$M) in culture medium were added to the apical chamber. In order to avoid bacteria settling on the bottom of the filter inserts and physically blocking transport of $AFB_1$ through the epithelial monolayer, cell culture dishes were placed on a plate shaker (shaking at 300 rpm) during incubations. Aliquots (15 $\mu$l) of culture medium were taken from both chambers at 0.5, 1, 24, 48, and 72 h after the addition of $AFB_1$ and GG. To quantify free $AFB_1$, $AFM_1$, and AFL, aliquots were centrifuged, and the supernatant was diluted with Milli-Q water when necessary and analyzed using high-performance liquid chromatography (HPLC). For the analysis of $AFB_1$ bound by GG in the apical chamber, the bacterial pellet was extracted twice with 0.5 ml methanol and analyzed by HPLC. The HPLC conditions used were as described previously (27). To assess the transport of $AFB_1$ through the monolayer, permeability coefficients (Pe) were calculated according to the following equation: $Pe = V_d/A \times \Delta\%/\Delta t$, where $V_d$ is the volume of the donor compartment, $A$ is the surface area of the monolayer, and $\Delta\%/\Delta t$ is the percentage of mass transported per second (26). The identities of metabolites ($AFM_1$ and AFL) were confirmed via spiking samples of cell culture medium with the respective aflatoxin standards. Amounts of metabolites were calculated by comparing peak areas of samples with peak areas of a standard.

**TER.** The integrity of the epithelial monolayer was assessed by measuring the electrical potential difference (transepithelial resistance [TER]) between the apical and basolateral chambers of the two-chamber model. Once Caco-2 cells form a confluent monolayer, they start differentiating and producing tight junctions, which results in an increase in TER. TER was monitored using an EVOM TER-meter (World Precision Instruments, Sarasota, FL). In untreated cells, the TER is known to increase until tight junction formation between cells is complete, which takes 21 days; TER levels then plateau for at least 8 days (31). TER readings were recorded over the first 21-day differentiation process, after induction with $1,25(OH)_2D_3$ and after the addition of either $AFB_1$ (150 $\mu$M) alone or GG ($1 \times 10^{10}$ or $5 \times 10^{10}$ CFU/ml) plus $AFB_1$ (150 $\mu$M) as described above. TER measurements were always conducted in fresh culture medium and after bacteria were removed by washing the Caco-2 monolayer with phosphate-buffered saline (five washes with 0.5 ml). Following the final TER measurement, cells were trypsinized and cell viability was assessed using a trypan blue exclusion assay. Cell viability was always high (>90% viability).

**$AFB_1$-induced DNA damage.** Differentiated Caco-2 cells were cultured in the presence of $1,25(OH)_2D_3$ for 5 days prior to $AFB_1$ exposure. Following 72 h of incubation with $AFB_1$ alone (150 $\mu$M) or with GG ($1 \times 10^{10}$ or $5 \times 10^{10}$ CFU/ml) and $AFB_1$ (150 $\mu$M), the cells were harvested using trypsin-EDTA and viability was assessed with trypan blue staining. Only cell cultures with viabilities of >90% were used for further assays. DNA damage was assessed using a DNA fragmentation assay. The DNA fragmentation assay includes lysis of the cells, extraction of DNA with phenol-chloroform, and gel electrophoresis as previously described in detail (24).

**Statistics.** SPSS 11.5 for Windows was used for the statistical analysis of the data. Results were subjected to the Mann-Whitney U test. Differences in mean values are considered significant at $P$ values of $\leq 0.05$.

## RESULTS

**$AFB_1$ binding.** In this study, the effect of nonviable probiotic bacteria on the transport, metabolism, and toxicity of $AFB_1$ in Caco-2 cells grown on a two-chamber dish was investigated. Following apical incubation of Caco-2 cells with either $AFB_1$ alone or GG plus $AFB_1$, samples were taken from the apical chamber to assess $AFB_1$ binding by GG and from the basolateral chamber to assess $AFB_1$ transport across the epithelium. $AFB_1$ bound by GG in the apical chamber was determined from the GG pellet after centrifugation (Fig. 1). GG binding of $AFB_1$ in the apical chamber was apparent by the first time point (0.5 h; data not shown) and was similar in magnitude to that at 1 h. At 1 h, GG at $5 \times 10^{10}$ CFU/ml had bound significantly ($P = 0.002$) more of the total $AFB_1$ ($61.0\% \pm 7.0\%$) than did GG at $1 \times 10^{10}$ CFU/ml ($40.1\% \pm 2.2\%$). After 24 h, there was a reduction in the total binding of $AFB_1$ to GG for both the higher GG concentration ($40.9\% \pm 2.2\%$ of total $AFB_1$) and the lower GG concentration ($18.0\% \pm 8.0\%$ of total $AFB_1$). There was no significant change in the amount of $AFB_1$ bound to GG at the later time points (data not shown). The overall mean recovery from each test well combining quantities within the media (apical and basolateral) and the pellet was 96.3%, though a number of samples at the earlier time point (1 h) had recoveries slightly greater than 100%. GG does not bind $AFB_1$ covalently, and for this reason the GG pellet could not be exhaustively washed to remove free $AFB_1$ within the pellet prior to the extraction of bound $AFB_1$. Particularly at high concentrations of apical $AFB_1$, as observed at the 1-h time point, this may have caused an overestimate of total $AFB_1$ recovery.

**$AFB_1$ transport.** The transport of $AFB_1$ to the basolateral side increased with time such that after 1 h $11.1\% \pm 1.9\%$ of total $AFB_1$ had transferred; this transport increased to $38.7\% \pm 5.5\%$ after 24 h (Fig. 1). After 48 and 72 h, there was no significant change in concentration. The amounts of $AFB_1$
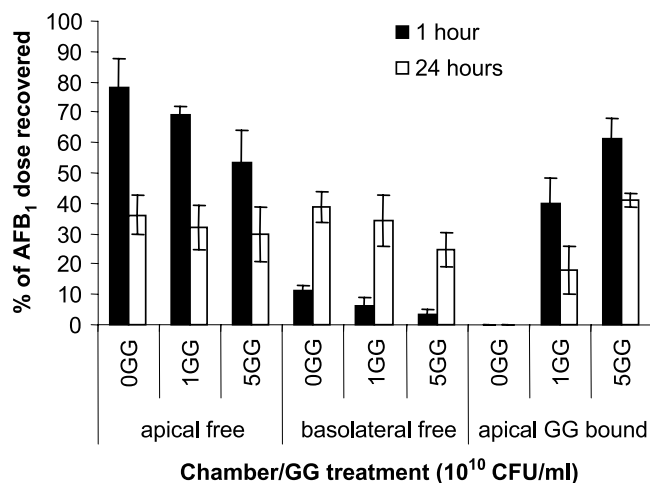
FIG. 1. Percentages of $AFB_1$ (150 μM) recovered free from culture media of apical and basolateral chambers and bound by 0, $1 \times 10^{10}$, or $5 \times 10^{10}$ CFU/ml GG in the apical chamber of the Caco-2 culture dish. Apical and basolateral culture media were removed after 1 or 24 h of incubation and centrifuged, and supernatant was injected into HPLC. The bacterial pellet of the apical chamber was extracted with methanol to remove $AFB_1$ bound by GG. A pellet was not obtained for the apical sample with no added GG. Values are presented as means of two replicates and two repeated experiments; error bars refer to standard deviation.

transported were significantly affected in the presence of GG, such that at 1 h the basolateral chamber contained less $AFB_1$ with both $1 \times 10^{10}$ CFU/ml GG (6.4% ± 2.5% of total $AFB_1$) and $5 \times 10^{10}$ CFU/ml GG (3.3% ± 1.8% of total $AFB_1$) ($P = 0.019$ and $P = 0.002$, respectively) than what was seen for $AFB_1$-only treatments. After 24 h, the reduction in $AFB_1$ transport was less pronounced and significant only in the presence of $5 \times 10^{10}$ CFU/ml GG (24.6% ± 5.6% [$P = 0.01$]). At later time points, $AFB_1$ transport remained stable (data not shown). A permeability coefficient was calculated to estimate the transport of aflatoxin through the monolayer (Fig. 2). The permeability of $AFB_1$ (24.6 × $10^{-6}$ ± 3.3 × $10^{-6}$ cm/s) was reduced slightly in the presence of $1 \times 10^{10}$ CFU/ml (17.2 ×
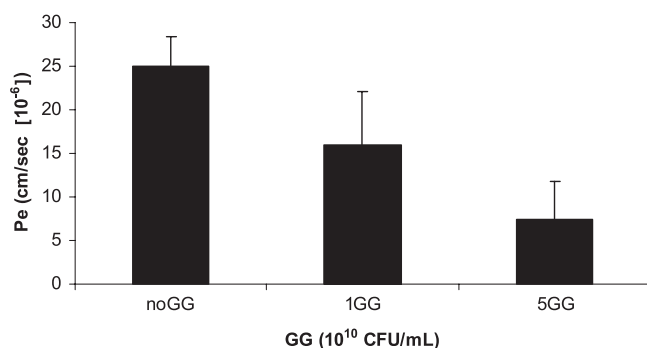


FIG. 2. Pe for $AFB_1$ (150 μM) transport through the Caco-2 monolayer calculated according to the following equation: Pe = $V_d/A \times \Delta\%/\Delta t$, where $V_d$ is the volume of the donor compartment, $A$ is the surface area of the monolayer, and $\Delta\%/\Delta t$ is the percentage of mass transported per second (27). Values are presented as means of two replicates and two repeated experiments; error bars refer to standard deviation.
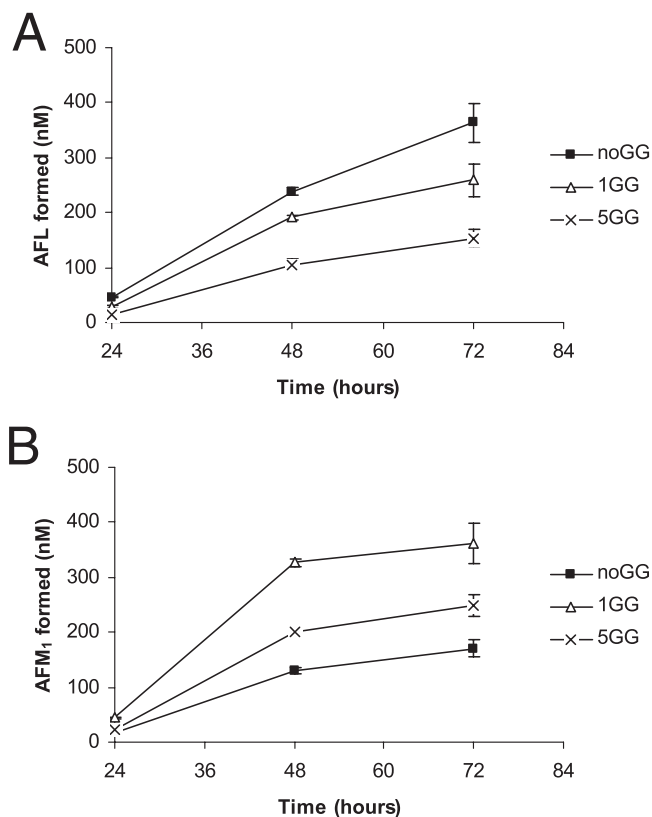


FIG. 3. Effect of heat-treated *Lactobacillus rhamnosus* strain GG ($1 \times 10^{10}$ [1GG] and $5 \times 10^{10}$ [5GG] CFU/ml) on the formation of AFL (A) and $AFM_1$ (B) in differentiated Caco-2 cells measured in culture medium of a basolateral chamber at three time points (24, 48, and 72 h) after $AFB_1$ addition (150 μM). Values are presented as means of three replicates and two repeated experiments; error bars refer to standard deviation.

$10^{-6}$ ± 6.1 × $10^{-6}$ cm/s [$P = 0.06$]) and significantly with 5 × $10^{10}$ CFU/ml (7.0 × $10^{-6}$ ± 4.8 × $10^{-6}$ cm/s [$P = 0.005$]) GG.

**Formation of free metabolites.** Following incubation with 150 μM $AFB_1$, the release of free metabolites into the culture medium on both the apical and basolateral chambers was also measured. Without bacteria present, $AFM_1$ and AFL were detectable in the culture media of both chambers, with basolateral concentrations of 17.4 ± 3.2 nM, 130.7 ± 5.9 nM, and 170.6 ± 16.5 nM for $AFM_1$ and 46.0 ± 1.2 nM, 238.0 ± 7.6 nM, and 363.2 ± 35.2 nM for AFL after 24, 48, and 72 h of incubation, respectively (Fig. 3A and B). There was a GG dose-dependent reduction in levels of AFL in both chambers (basolateral only shown in Fig. 3A) at all time points. After 72 h of incubation, basolateral AFL concentration was reduced significantly to 259.3 ± 30.2 nM ($P = 0.004$) with $1 \times 10^{10}$ CFU/ml GG and to 153.1 ± 15.6 nM ($P = 0.002$) with $5 \times 10^{10}$ CFU/ml GG from what was seen for $AFB_1$-only treatment (363.2 ± 35.2 nM). Surprisingly, the basolateral $AFM_1$ concentration was increased after 72 h of incubation from 170.6 ± 16.5 nM with $AFB_1$ only to 361.8 ± 36.0 nM ($P = 0.002$) in the presence of $1 \times 10^{10}$ CFU/ml GG and to 248.2 ± 20.4 nM in the presence of $5 \times 10^{10}$ CFU/ml GG (significantly [$P = 0.01$] lower than what was seen for $1 \times 10^{10}$ CFU/ml GG) (Fig. 3B).
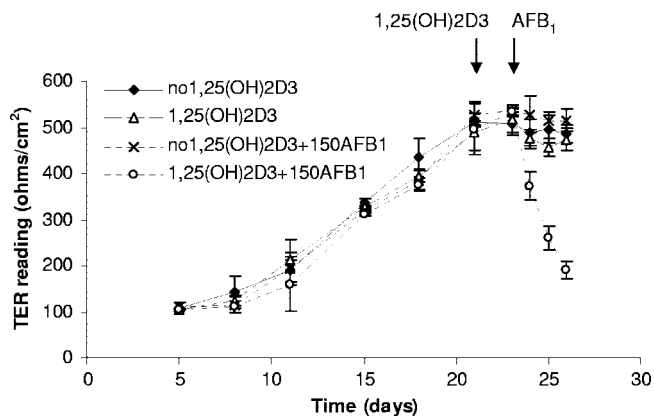
FIG. 4. Change in TER during 21-day differentiation of Caco-2 cells and induction with 1,25(OH)$_2$D$_3$ for 2 days and following incubation with AFB$_1$ (150 μM) (150AFB1) for 3 days. TER was measured in fresh culture media of apical and basolateral chambers. Values are presented as means of three replicates and two repeated experiments; error bars refer to standard deviation.



FIG. 5. Effect of heat-treated *Lactobacillus rhamnosus* strain GG (1 × 10$^{10}$ CFU/ml) on AFB$_1$ (150 μM)-induced reduction in TER in differentiated Caco-2 cells [following induction with 1,25(OH)$_2$D$_3$ for 48 h]. TER was measured in fresh culture medium. Values are presented as means of three replicates and two repeated experiments; error bars refer to standard deviation.

**Intestinal cell toxicity.** To assess the toxic effect of AFB$_1$ on Caco-2 cells, we studied the integrity of the cell monolayer, by use of TER measurements, and AFB$_1$-induced DNA damage. TER readings increased over 3 weeks of differentiation of the Caco-2 cells and reached a plateau towards day 21 (502 ± 40 Ω/cm$^2$) (Fig. 4). Further incubation of cells with or without 1,25(OH)$_2$D$_3$ for 2 days had no significant effect on the TER (488 ± 28 and 496 ± 20 Ω/cm$^2$), and further growth for 3 days in the absence of AFB$_1$ or after adding AFB$_1$ to cells without prior 1,25(OH)$_2$D$_3$ preactivation resulted in no significant change in TER. In Caco-2 cells induced with 1,25(OH)$_2$D$_3$ for 2 days and subsequently incubated with 150 μM AFB$_1$, significant decreases in TER by 31.1% ± 3.3% ($P = 0.01$), 52.0% ± 2.9% ($P = 0.004$), and 64.4% ± 5.2% ($P < 0.001$) were observed at 24, 48, and 72 h after AFB$_1$ addition, respectively. When bacteria were coincubated with AFB$_1$, the TER reduction caused by AFB$_1$ alone was significantly attenuated. With 1 × 10$^{10}$ CFU/ml GG after 24 h of incubation, the TER was reduced by 18.4% ± 2.1%, compared to 31.1% ± 3.3% without GG ($P = 0.002$), and after 48 h the TER was reduced by 48.8% ± 1.6%, compared to 52.0% ± 2.9% without GG ($P = 0.04$) (Fig. 5). Higher bacterial numbers and longer incubation times were not suitable for this assay, since greater quantities of bacteria influenced TER readings, independent of the presence of AFB$_1$ (data not shown).

**DNA damage.** As a marker of AFB$_1$-induced DNA damage in the cells, DNA fragmentation of Caco-2 cells exposed to AFB$_1$ following 1,25(OH)$_2$D$_3$ activation for 5 days was assessed using the DNA fragmentation assay. DNA fragmentation was observed for cells treated with 150 μM AFB$_1$ for 72 h (Fig. 6, lane 2) but not for cells treated with AFB$_1$ and GG (1 × 10$^{10}$ or 5 × 10$^{10}$ CFU/ml) (Fig. 6, lanes 3 and 4), or with GG alone (5 × 10$^{10}$ CFU/ml) (Fig. 6, lane 5).

## DISCUSSION

Following oral exposure, AFB$_1$ is readily absorbed in the gut and reaches the liver and systemic circulatory system. In vitro cell culture models have been u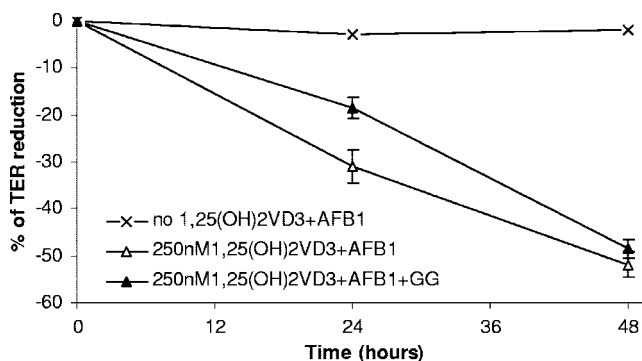sed to assess the bioavailability and transport of several mycotoxins (1, 26, 45). In our study, we used the Caco-2 cell culture model to study AFB$_1$ transport and intestinal toxicity. We confirmed published results that AFB$_1$ is transported rapidly through the intestinal cell monolayer and that permeability coefficients of AFB$_1$ are in agreement with the literature (26, 45). The transport occurred in a time-dependent manner, though no further increase in the basolateral concentration of AFB$_1$ occurred after 24 h. The amount of AFB$_1$ transported to the basolateral chamber was significantly reduced when GG was added to the apical compartment. The reduction in transported AFB$_1$ was also dependent on GG dose and at both doses of GG reached a plateau at 24 h. At the same time, additional AFB$_1$ was recovered from the bacterial pellet, indicating that GG was able to bind AFB$_1$ in this cell culture system and hence to reduce AFB$_1$ transport through the intestinal cell monolayer. The binding of AFB$_1$ by GG was initially high, with 40.1% ± 8.3% and 61.0% ± 7.0% of the total AFB$_1$ being bound in the first hour for the lower and higher GG concentrations, respectively, but over the
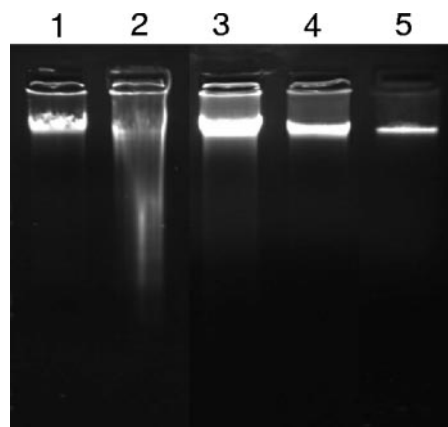


FIG. 6. DNA fragmentation in 1,25(OH)$_2$D$_3$-induced Caco-2 cells caused by AFB$_1$ (150 μM) and GG. Lanes: 1, untreated cells; 2, cells treated with AFB$_1$ for 72 h; 3, cells treated with AFB$_1$ and GG (1 × 10$^{10}$ CFU/ml); 4, cells treated with AFB$_1$ and GG (5 × 10$^{10}$ CFU/ml); 5, cells treated with GG (5 × 10$^{10}$ CFU/ml) only.

course of the study binding to GG was reduced to 18.0% ± 8.0% and to 40.9% ± 2.2%, respectively, after 24 h. This suggests that $AFB_1$ is reversibly bound to the bacterial surface (24) and that as aflatoxin is transported to the basolateral chamber, the equilibrium between bound and free $AFB_1$ in the apical chamber adjusts until the overall system is in equilibrium. The reduced transport of $AFB_1$ in the presence of GG in this model is in agreement with our ex vivo findings showing that GG reduced $AFB_1$ transport in duodenal loops (9). These data indicate that the Caco-2 model is useful to study the effect of bacterial binding on $AFB_1$ absorption and could be further developed to study the role of food matrix in $AFB_1$ uptake and bacterial binding (45).

The metabolism and toxicity of $AFB_1$ have been studied with human cellular systems derived from liver (21, 43) or respiratory tract (44), but the impact of the intestinal metabolism and toxicity has not been fully investigated. We were able to detect $AFM_1$ and AFL in the culture media of both the apical and basolateral chambers after incubating the apical chamber with $AFB_1$, albeit at relatively low concentrations compared to the amount of $AFB_1$ added. Total formation of $AFM_1$ and that of AFL in both chambers after 72 h were only 0.4 and 0.8% of the $AFB_1$ dose, respectively, and this likely reflects the low activity of the relevant enzymes. $AFQ_1$, a metabolite generated by CYP 3A4, was not evident in the culture media. This was surprising, as the culture model included $1,25(OH)_2D_3$ to up-regulate CYP 3A4 expression (35). The lack of detection of this metabolite may reflect the detection limit for $AFQ_1$, which is considerably (approximately 100-fold) higher than that for $AFM_1$ (27). However, the fact that toxicity of $AFB_1$ was observed only for $1,25(OH)_2D_3$-pretreated cells is strongly supportive of an increased CYP 3A4 activity and generation of the highly reactive aflatoxin epoxide. In the presence of GG, the level of AFL in culture media was reduced, as would be predicted for a model that restricted $AFB_1$ bioavailability. AFL can be readily oxidized back to $AFB_1$ and has therefore been suggested as a "reservoir" for $AFB_1$ in vivo rather than a detoxification product (5). The reduced level of this metabolite may therefore be relevant to the toxicity caused by $AFB_1$. On the contrary, $AFM_1$ levels in culture media increased in the presence of $1 \times 10^{10}$ CFU/ml GG rather than decreased as would be expected after reduced cellular uptake of $AFB_1$. Caloni and colleagues (1) recently reported that differentiated Caco-2 cells are particularly able to accumulate $AFM_1$ from culture media such that the medium concentration can be reduced by up to 95% of the initial concentration. This property of $AFM_1$ may in part explain the high frequency of its distribution into breast milk compared to that of other aflatoxin metabolites (30). It has also been shown that GG can effectively bind $AFM_1$ (29). It is therefore possible that the equilibrium between $AFM_1$ within the cell and that which is released into the medium, which usually favors cellular accumulation (1), is being disrupted due to the presence of GG in the medium. It is of note that $AFM_1$ levels in the media subsequently decreased at the higher GG concentration from the level seen for the low GG concentration, further suggesting that GG is able to sequester the metabolite in this model. However, this study was not able to confirm if alterations in the levels of these metabolites reflect alterations in $AFB_1$ availability due to GG binding or subsequent sequestering of the

metabolites within the culture medium by GG or a combination of both processes. We previously observed increased fecal excretion of $AFM_1$ in rats dosed with live GG (15); thus, the possible impact of probiotic bacteria on $AFM_1$ formation or mobilization warrants further clarification.

The effects of GG on restricting transport of $AFB_1$ would clearly have a positive effect on reducing the bioavailability and therefore the burden of aflatoxin-induced damage within the liver and associated hepatocarcinogenic risk. Chronic aflatoxin exposure has also been associated with growth faltering (12, 13), and in independent studies from the Gambia, intestinal enteropathy, characterized by "intestinal leakiness," has been associated with early childhood growth faltering (2, 25). Given that (i) enterocytes have the metabolic enzymes to generate the reactive aflatoxin epoxide (4), (ii) aflatoxin exposure is frequent at high levels (38, 39) in regions reporting an association between growth faltering and intestinal leakiness (2, 25), and (iii) aflatoxin exposure has been associated with infant growth faltering (13, 14, 39), it is plausible that observations of aflatoxin-associated growth faltering may be explained at least in part by a direct action of the aflatoxin epoxide within the enterocytes. To assess this direct toxic effect of $AFB_1$ on the Caco-2 monolayer, we studied its effect on monolayer integrity, using TER measurements. TER is a measure of epithelial barrier function, with higher TER values indicating intact tight junction complexes. The TER of the Caco-2 monolayer increased until a steady state was maintained after about 21 days (34). A decrease in TER is suggestive of damage to the tight junctions, which form part of the scaffolding between epithelial cells. Damage at this site makes the monolayer barrier more susceptible to the unregulated passage of solutes, or "leaky." In this study, $AFB_1$ caused a time-dependent decrease in TER if cells were induced with $1,25(OH)_2D_3$. In the absence of $1,25(OH)_2D_3$ pretreatment, there was no effect with $AFB_1$ incubation, indicating the requirement for CYP 3A4 induction to generate sufficient quantities of the reactive aflatoxin epoxide. The reduction in TER was not associated with reduced cell viability, suggesting that $AFB_1$ might act directly or indirectly on the protein scaffolding networks that maintain cell monolayer integrity. A recent study with rats suggests that the targeting of secretory proteins in hepatocytes, which are important structural and functional components of plasma membranes, was inhibited by $AFB_1$ (36). It will be of interest to further assess aflatoxin-induced damage within the intestine in terms of junctional complex stability and barrier function, because damage to the integrity of the barrier may contribute to the intestinal enteropathy or "leakiness" observed by Lunn and colleagues (2, 25) in Gambian infants. In the presence of GG ($1 \times 10^{10}$ CFU/ml), the $AFB_1$-induced decrease in TER was significantly restricted, suggesting that the bacteria were able to reduce $AFB_1$ availability. Higher bacterial numbers ($5 \times 10^{10}$ CFU/ml) could not be used in this assay due to direct influence by the bacteria on TER readings.

The genotoxicity of $AFB_1$ has been widely studied by use of hepatic cells with the Comet assay (39, 40, 43), but data from intestinal enterocytes are limited. $AFB_1$-DNA adducts have been detected in animal and human intestinal samples (4, 22), but to our knowledge only one study in rat intestine investigated DNA damage (46). In the latter study, DNA damage was observed only in vitro, in isolated rat jejunal cells, but not in

rats dosed with AFB$_1$ in vivo. In our study, we used DNA fragmentation as a marker of AFB$_1$-induced DNA damage in differentiated Caco-2 cells exposed to AFB$_1$ following induction of CYP 3A4. DNA fragmentation was assessed by extracting DNA and separating intact and damaged DNA by use of gel electrophoresis. DNA damage was apparent following treatment with AFB$_1$, while coincubation with GG reduced the AFB$_1$-induced damage in this test system, further indicating the protective role of GG.

Our data indicate that the Caco-2 model is suitable for AFB$_1$ transport studies evaluating the effect of probiotics. Nonviable bacteria were used because live bacteria multiply in the cell culture medium, affecting pH and nutrient availability. Nonviable GG bacteria have been shown to have an AFB$_1$ binding capacity comparable to that of live GG bacteria (7), and thus this does not restrict their use in model systems. In fact, a major issue in the field of probiotic research is the survival of numbers of bacteria sufficient to cause colonization or to exert a beneficial physiological effect within the gut. Therefore, the possibility of using GG in a nonviable format to restrict aflatoxin uptake may be advantageous. One limitation in this study was that AFB$_1$ was applied at a relatively high concentration and for a long time (up to 72 h). Based on the levels of CYP 3A4 induction by 1,25(OH)$_2$D$_3$ described in the literature (11, 35), it is likely that the levels of CYP 3A4 in our cell model are somewhat below those in intestinal cells in vivo. The use of a metabolically more active Caco-2/TC7 clone which expresses CYP 3A4 (34) might help overcome this problem for future studies. It is always difficult to directly translate the effects from a cell culture model to a role within humans. We would expect GG to colonize mainly the large intestine following repeated administration, but it might also adhere within the upper intestinal tract. The upper intestine is the major site for AFB$_1$ absorption (34); thus, colonization of the intestinal track may impart a benefit in terms of restricting aflatoxin bioavailability. Approximately 50% of AFB$_1$ metabolites from the liver will be excreted in the feces (mainly as AFB$_1$-glutathione conjugate) via the bile (20). Release of the parent aflatoxin following colonic microflora activity can allow the reuptake and circulation of the parent compound. It is therefore possible that GG colonization of the large intestine will in part restrict this recirculation and shuttle GG-bound aflatoxin to be excreted via the fecal route. However, we anticipate that the maximum benefit will occur if GG is consumed immediately before or at the same time as aflatoxin-contaminated foods are consumed. This will allow the greatest opportunity for interaction within the gut lumen.

In summary, our results clearly show that AFB$_1$ transport can be reduced by GG binding in the Caco-2 model. We found that 1,25(OH)$_2$D$_3$-induced Caco-2 cells can be used to estimate the intestinal metabolism and toxicity of AFB$_1$ by use of TER measurements. The data suggest that bacterial binding of AFB$_1$ will impact on bioavailability and therefore reduce the burden of toxicity. Amounts of GG are in line with quantities typically found in probiotic yogurts. It is important to note that the effects of GG were obtained with heat-inactivated bacteria; thus, there are no concerns of effective bacterial survival for efficacy. However, the possible role of live probiotics on intestinal AFB$_1$ metabolism and toxicity still needs to be investigated.

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