Probiotic supplementation reduces a biomarker for increased risk of liver cancer in young men from Southern China[1–3]

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ABSTRACT

Background: In vitro and in vivo studies suggest that selected strains of probiotic bacteria can form tight complexes with aflatoxin B_1 and other carcinogens.

Objective: The aim of the present study was to determine whether administration of probiotic bacteria could block the intestinal absorption of aflatoxin B_1 and thereby lead to reduced urinary excretion of aflatoxin B_1-N^7-guanine (AFB-N^7-guanine), a marker for a biologically effective dose of aflatoxin exposure. Elevated urinary excretion of this aflatoxin-DNA adduct is associated with an increased risk of liver cancer.

Design: Ninety healthy young men from Guangzhou, China, were randomly assigned to 2 groups; one group received a mixture of *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* strains 2 times/d for 5 wk, and the other group received a placebo preparation. The subjects provided 4 urine samples: at baseline, at 3 and 5 wk after starting the supplementation, and at the end of the 5-wk postintervention period.

Results: The percentage of samples with negative AFB-N^7-guanine values tended to be higher in the probiotic group than in the placebo group during the 5-wk intervention period (odds ratio: 2.63, $P = 0.052$), and a statistically significant decrease in the concentration of urinary AFB-N^7-guanine was observed in the probiotic group. The reduction was 36% at week 3 and 55% at week 5. The geometric means for the probiotic and placebo groups were 0.24 and 0.49 ng AFB-N^7-guanine/mL, respectively, during the intervention period ($P = 0.005$).

Conclusion: A probiotic supplement reduces the biologically effective dose of aflatoxin exposure and may thereby offer an effective dietary approach to decrease the risk of liver cancer. *Am J Clin Nutr* 2006;83:1199–203.

KEY WORDS Probiotic bacteria, aflatoxins, aflatoxin B_1-N^7-guanine, supplementation, young men, liver cancer

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) varies widely worldwide. In men, the highest incidence rates are found in eastern Asia, particularly in China, where HCC is the third most common cause of cancer death (1). Chronic infection with the hepatitis B virus (HBV) is the strongest risk factor for HCC worldwide (2, 3). However, populations with a similar prevalence of HBV infection have different incidences of HCC (3), which suggests the presence of other important risk factors.

Aflatoxins, a group of mycotoxins produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus*, are established human hepatocarcinogens (4–6) and are well-known HCC risk factors when present in foodstuffs (2, 7). They play an important role in modifying the risk of liver cancer associated with HBV. After being metabolized in the liver, the toxin can bind to guanine in DNA, resulting in mutations at codon 249 of the *TP53* tumor suppressor gene (8). In addition to being potent carcinogens, aflatoxins are cytotoxic, and associations between childhood aflatoxin exposure and growth faltering (9–12) and reduced concentrations of salivary IgA (12) have been reported. This highlights the need to reduce or eliminate exposure to aflatoxin (13). The approach to prevent exposure to aflatoxins has been to ensure that foods consumed have the lowest possible aflatoxin concentrations. Although this is achieved in developed countries via strict food regulations, it has clearly failed as a control measure in developing countries where the problem is more evident. Additional prevention strategies, such as chemo- or dietary prevention, need to be considered in the high-risk regions of developing countries. These strategies need to be completely safe, inexpensive, and mechanistically simple. Chemopreventive agents (oltipraz and chlorophyllin), which reduce the burden of harmful aflatoxin metabolites in the body, have been studied and shown to be potentially beneficial in targeted groups (14, 15).

Our previous work with >250 strains of lactic acid bacteria isolated from either dairy products or healthy human microbiota showed that 2 *Lactobacillus rhamnosus* strains, LGG and LC705 (both possess probiotic properties), were the most efficient strains in binding a range of mycotoxins, including aflatoxins

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(16–19). A single viable bacterium is able to bind $>10^7$ aflatoxin B_1 (AFB_1) molecules, and binding appears to occur on the bacterial surface predominantly by hydrophobic interactions between the AFB_1 molecules and the carbohydrate and protein components of the bacterial cell wall (20). The in vitro results led to an investigation of the ability of these strains to bind AFB_1 in vivo and to test whether the strength of binding was sufficient to reduce AFB_1 bioavailability. Both *Lactobacillus rhamnosus* strains GG and LC705 bound AFB_1 and reduced its absorption (74% reduction in the uptake of AFB_1 by the duodenal tissue) from ligated duodenal loops of 1-wk-old chickens (21).

A pilot trial conducted in Egypt involving 20 volunteers, of whom 10 were given capsules containing lyophilized *Lactobacillus rhamnosus* strain LC705, showed that the probiotic supplement significantly reduced fecal concentrations of free AFB_1 (22). However, AFB_1 bound by the fecal material could not be recovered, and, therefore, these data did not provide a direct measure of AFB_1 absorption from the gastrointestinal tract. Consequently, the primary goal of the present study was to determine whether administration of probiotic bacteria could prevent or reduce the absorption of AFB_1 from the small intestine. Urinary excretion of aflatoxin B_1-N^7-guanine (AFB-N^7-guanine) was used as a marker for a biologically effective dose of AFB_1. The elevated urinary excretion of this aflatoxin–DNA adduct is associated with an increased risk of liver cancer (7).

SUBJECTS AND METHODS

Study population

The study population was from Guangzhou, Guangdong province, which is located in southern China, where exposure to aflatoxin via food ingestion is common. Male students ($n = 300$) at the Sun Yat-Sen University were screened for the presence of the hydroxylated metabolite of AFB_1, aflatoxin M_1 (AFM_1), in a spot urine sample. Of those with a detectable concentration of AFM_1 (>0.008 ng/mL), 90 were selected for the intervention. Because the subjects were selected on the basis of detectable urinary AFM_1 concentrations, they do not represent the general aflatoxin exposure of the population in the region but that of an exposed group.

The subjects underwent a complete health examination, including the following: medical history, smoking status, physical examination, routine hematological and clinical biochemistry tests on hepatic and renal function, and tests of antigens to hepatitis B virus (14 subjects tested positive for the hepatitis B virus). The clinical chemistry measurements and hepatitis B surface antigen determination were carried out at the Sun Yat-Sen University according to standard protocols. Exclusion of subjects was based on abnormal hematological values and outlying AFM_1 concentrations (<0.008 or >20 ng/mL). Consumption of dietary sources of aflatoxins was assessed with the use of a food-frequency questionnaire. All subjects resided in student accommodations and consumed their main meals (breakfast, lunch, and dinner) at the student restaurant operated by the university. Antibiotics or traditional Chinese medicines and herbs, or both, had been used during the preceding 2 mo by 21 students (25%) who were evenly distributed between the 2 study groups. The protocol was approved by the Ethical Committees at Kuopio University (Finland) and Sun Yat-Sen University (China). The procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Study design

The study design was a randomized, double-blind, placebo-controlled trial with 2 parallel groups. The subjects were randomly assigned to 2 groups ($n = 45$ per group); one group received the probiotic preparation and the other received a placebo preparation. The subjects received written and oral directions to take 2 capsules per day immediately before main meals (breakfast and dinner). The probiotic preparation contained a mixture of the strains *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* (1:1, wt:wt) at a dose of $2–5 \times 10^{10}$ colony-forming units/d. The placebo capsules contained only cellulose. The probiotic strains used are of a grade that is generally recognized as safe and are commonly used in the manufacturing of various dairy products.

During the 5-wk intervention period, the subjects consumed their normal diets. Intake of dietary sources of aflatoxins was assessed with the use of a structured food-frequency questionnaire. The subjects provided one early morning urine sample in weeks 3 and 5 of the intervention period and after a 5-wk postintervention period. Compliance to the study protocol was assessed based on the presence of *Lactobacillus rhamnosus* LC705 in fecal samples taken at the same time points as the urine samples.

Sample size rationale

The sample size of 90 subjects in total with 45 subjects in each group was based on the assumption that intervention with probiotic bacteria can reduce the concentration of AFB-N^7-guanine by ≥25% when compared with placebo, giving a standardized effect of change = 0.5. With these assumptions, the required sample size for 2-sided significance of 5% and power of 80% was 86 subjects per group. By using 0.5 as the correlation between repeated AFB-N^7-guanine measurements (baseline + 2 intervention measurements + postintervention), the required number of subjects is reduced to 33 per group (23). The 2 phases of the study (intervention and postintervention) are considered separate phases. To compensate for the anticipated dropout rate (20%), a total of 90 subjects were randomly assigned, 45 in each group.

Aflatoxin analyses

Urinary concentrations of AFB-N^7-guanine were measured as described by Mykkänen et al (24) Briefly, a 25-mL aliquot was acidified with 1 mol hydrochloric acid/L and 1 mol ammonium formate/L. After centrifugation at $3000 \times g$ for 15 min at 4 °C, the acidified sample was passed through a Bond Elut C18 cartridge (Varian, Middelburg, Netherlands), and the cartridge was washed with milli-Q water (Millipore, Espoo, Finland) and 5% methanol, followed by washes with 10% basic acetonitrile (ammonia:acetonitrile:water, 1:10:90) and 10% acidic acetonitrile (acetic acid:acetonitrile:water, 1:10:90). AFB-N^7-guanine was eluted from the cartridge with 40% acidic acetonitrile (acetic acid:acetonitrile:water, 1:40:60), the eluant was extracted twice with 2 volumes of dichloromethane, and the extracts were pooled and dried in a vacuum. The vacuum-dried extracts were reconstituted in 30% acetonitrile:methanol (1:1, by vol) in 20 mmol ammonium acetate/L buffer (pH 3.9) for HPLC analysis. Reverse-phase HPLC, a Shimadzu model SPD-10 Avp UV-Vis

detector in series with Shimadzu RF-10AXL fluorescence detector (excitation 366 nm, emission 440 nm; Shimadzu, Kyoto, Japan) was used to quantify aflatoxins. The HPLC column used was the ODS Spheri-5 Brownlee column (220 × 4.6 mm, 5 μm; Perkin Elmer, Shelton, CT) fitted with a C_{18} guard column (Perkin Elmer). Chromatographic separation was obtained by an isocratic gradient over 40 min with a mobile phase of 30% acetonitrile:methanol (1:1, by vol) in 20 mmol ammonium acetate/L buffer (pH 3.9) at a flow rate of 1.5 mL/min. The assay temperature was 40 °C, and the sample volume injected was 10 μL. Standard solutions of AFB-N^7-guanine were eluted at 4.6 min. The limit of detection was 0.005 ng AFB-N^7-guanine/mL urine. To ensure the correctness of the HPLC results, we applied a strict quality control system by measuring the recovery and reproducibility of the assay. Samples were run in batches with authentic standards running between every 10 samples to control for any changes to the retention time.

Statistical analysis

Urinary AFB-N^7-guanine during the 5-wk intervention period was the primary variable. The study groups were compared during the intervention period at week 3 and week 5. In addition, a comparison was made 5 wk after the cessation of intervention to find out if the urinary AFB-N^7-guanine concentration returned to the baseline value. Urinary AFB-N^7-guanine was first dichotomized (negative or positive). Adjusted ORs, where the corresponding baseline urinary AFB-N^7-guanine was included as a categorical covariate, were calculated from logistic regression models separately for each time point. Because each subject contributed to 2 binary outcomes during treatment, we fit models using Generalized Estimating Equation methods (25) as a primary analysis, assuming unstructured covariance matrix and a logit link. We started with a model that included treatment, baseline AFB-N^7-guanine, time, and the interaction between treatment and time as factors. The factors were retained in the model if they were significant at the 0.10 level. The time-effect and the interaction between treatment and time were not significant ($P = 0.280$ and $P = 0.860$, respectively) and were excluded from the final model. The final model was used to estimate the OR and the CI.

Because of the high rate of nondetectable values (ie, values below the detection limit), the analysis of mean concentrations of urinary AFB-N^7-guanine was conducted as a secondary analysis. Zero concentrations were transformed to the observed minimum value divided by 2. The final distributions of AFB-N^7-guanine were skewed to the right, and, therefore, all values were logarithmically (ln) transformed before analysis. Analysis of covariance was applied with respect to the level of AFB-N^7-guanine concentrations in urine at weeks 3 and 5 and after the cessation of intervention, separately. The corresponding ln-transformed baseline concentration was included as a continuous covariate. The baseline-adjusted group means and their differences were then back-transformed to the original units and the results are given as geometric means and ratios (probiotic:placebo) with 95% CIs, respectively. To account for multiple comparisons in repeated measurements, the urinary concentration of AFB-N^7-guanine was analyzed with the use of an analysis of variance (ANOVA) for repeated measures. Concentrations at weeks 3 and 5 were included as dependent variables, and the baseline concentration was included as a continuous covariate. ANOVA for repeated measures was applied to study the difference between study groups, the time-effect (ie, the change during the intervention period), and the interaction between treatment and time. The data were analyzed with SPSS version 12.0 (SPSS Inc, Chicago, IL) and SAS version 9.1.3 (SAS Institute, Cary, NC).

RESULTS

According to blood biochemistry outcome, the students were healthy. The analysis of blood α-fetoprotein indicated that none of the subjects had inflammatory liver disease. In addition, the white blood cell and blood lymphocyte counts fell within the normal respective reference ranges ($4–10 \times 10^9$/L for white blood cells and $0.6–4.1 \times 10^9$/L for blood lymphocytes), indicating that the subjects did not have chronic inflammation. Eleven subjects (5 in the probiotic group and 6 in the placebo group) that were included in the study tested positive for hepatitis B antigen. The intervention groups did not differ significantly with respect to baseline concentrations of AFB-N^7-guanine (median: 0.34 and 0.55 ng AFB-N^7-guanine/mL urine for the probiotic and placebo groups, respectively), and there were no significant differences in the intakes of food groups known to be the most common sources of dietary aflatoxins in China between the probiotic and placebo groups (**Table 1**).

Adherence to the study protocol, which was assessed based on ingestion of the probiotic capsule and collection of urine samples, was satisfactory. The ingestion of the probiotic capsule was confirmed by the concentration of *Lactobacillus rhamnosus* LC705 in fecal samples. In the subjects who received the probiotic mixture, *Lactobacillus rhamnosus* LC705 constituted the major part of the fecal *Lactobacillus* population, whereas in the placebo group this bacteria was absent. Overall, 90% of the subjects (87% of the probiotic group and 93% of the placebo group) contributed all urine samples.

No significant difference in the percentage of detectable AFB-N^7-guanine values was seen between the treatment groups at baseline [51% (20 of 39 samples) of probiotic and 60% (25 of 42 samples) of placebo samples]. The relatively high percentage of nondetectable values in the placebo group during the baseline, intervention, and postintervention periods (≈39%) may reflect both an insufficient analytic sensitivity to low exposure and a lack of recent exposure to aflatoxins in meals. However, the percentage of samples with negative values tended to be higher

TABLE 1
Weekly intake of major foods in the diet of healthy Chinese male students who received either a probiotic or a placebo preparation[1]

Foods (servings)	Probiotic group (n = 39)	Placebo group (n = 44)
Rice (scoops)	157 ± 36	164 ± 35
Bread (slices)	9.9 ± 5.1	10.0 ± 4.6
Pork (chops)	6.5 ± 4.7	6.9 ± 3.8
Chicken (chops)	6.4 ± 4.4	4.7 ± 4.1
Beef (chops)	2.8 ± 4.3	2.8 ± 4.0
Fish (chops)	2.1 ± 3.3	2.2 ± 3.6
Beans (scoops)	5.5 ± 2.8	5.1 ± 2.9
Noodles (scoops)	1.3 ± 1.8	1.5 ± 2.1
Peanuts, total (handfuls)	0.4 ± 1.0	0.3 ± 0.6

[1] All values are $\bar{x}$ ± SD. No difference in the intakes of food groups known to be the most common sources of dietary aflatoxins in China were observed between the probiotic and placebo groups, $P > 0.05$ (Mann-Whitney U test).

TABLE 2
Effect of probiotic intervention on the proportion of urinary aflatoxin B_1-N^7-guanine values below the detection limit in healthy Chinese men[1]

	Probiotic group ($n = 39$)	Placebo group ($n = 44$)	Probiotic compared with placebo group OR (95% CI)[2]	*P*
	% negative	*% negative*		
Baseline	48.7	40.5		
Intervention				
Week 3	56.4	35.7	2.88 (0.89, 9.39)	
Week 5	61.5	42.9	2.43 (0.78, 7.61)	
Total[3]			2.63 (0.99, 6.95)	0.052
Postintervention period	38.5	45.2	0.58 (0.21, 1.59)	0.289

[1] *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* (1:1, wt:wt) at a dose of 2–5 × 10^{10} colony-forming units/d. OR, odds ratio.
[2] ORs were calculated with the use of logistic regression analysis, where baseline aflatoxin B_1-N^7-guanine is included as a categorical covariate.
[3] Data was corrected by generalized equation equations before analysis.

in the probiotic group than in the placebo group during the 5-wk intervention period (OR: 2.63, $P = 0.052$) (**Table 2**). That effect of the probiotic was not seen at the postintervention visit 5 wk after the cessation of intervention; the difference between groups was not significant ($P = 0.289$).

Probiotic administration led to a statistically significant decrease in the level of urinary excretion of AFB-N^7-guanine. The reduction was 36% at week 3 and 55% at week 5, but disappeared during the 5-wk postintervention period (**Table 3**). The probiotic-to-placebo ratios at weeks 3 and 5 of the intervention were significantly below one. Adjustment for HBV status did not significantly influence the results. In an ANOVA for repeated measures, the geometric mean during the intervention period for the probiotic group was 0.24 ng AFB-N^7-guanine/mL and that for the placebo group was 0.49 ng AFB-N^7-guanine/mL, and the probiotic to placebo ratio was 0.49 (95% CI: 0.30, 0.80; $P = 0.005$). After the cessation of intervention, the difference between groups was not significant ($P = 0.703$).

DISCUSSION

Our results clearly showed that it is possible to reduce the biologically effective dose of aflatoxin by giving subjects who had detectable aflatoxin exposure a probiotic twice a day for 5 wk. The study was designed to have the statistical power to evaluate biomarker modulation with the use of repeated samples. Thus, the power to detect biomarker modulation was amplified considerably beyond that afforded by a single cross-sectional intergroup comparison. The significance of this reduction is that the presence of AFB-N^7-guanine adducts in urine may reflect several outcomes in addition to dietary exposure to aflatoxins. First, strong dose-response relations with this biomarker have been observed in animals and humans exposed to different amounts of aflatoxin (26, 27). Second, because these adducts are markers for promutagenic lesions, their presence indicates that damage to the genome has occurred. Third, prospective, nested case-control studies that initially characterized the strong chemical–viral interaction in the cause of HCC have clearly shown that elevated concentrations of this DNA-adduct biomarker are specifically associated with an increased risk of liver cancer (7). Lastly, diminished concentrations of AFB-N^7-guanine adducts have been associated with a reduced risk of HCC in several chemoprevention studies in animals (28–30).

The observed 55% reduction in the geometric mean excretion of AFB-N^7-guanine in the subjects who consumed probiotic bacteria is consistent with the reduction obtained (49%) in the chlorophyllin intervention study conducted in Qidong, China (15). However, the reduction was achieved faster with probiotic bacteria (5 wk after the supplementation) than with chlorophyllin intervention (12 wk after supplementation).

Loeb (31) predicted that a 2-fold reduction in mutation rates (as might be anticipated from the decline in DNA adduct burden

TABLE 3
Effect of probiotic intervention on urinary excretion of aflatoxin B_1-N^7-guanine in healthy Chinese men[1]

	Probiotic group ($n = 39$)	Placebo group ($n = 44$)	Probiotic:placebo[2]	*P*
	ng/ml	*ng/ml*		
Baseline	0.42 (0.22, 0.82)	0.54 (0.29, 1.03)		
Intervention				
Week 3	0.27 (0.15, 0.47)	0.63 (0.34, 1.16)	0.51 (0.29, 0.89)	
Week 5	0.19 (0.11, 0.31)	0.46 (0.25, 0.86)	0.48 (0.27, 0.84)	
Total[3]	0.24 (0.17, 0.34)	0.49 (0.35, 0.69)	0.49 (0.30, 0.80)	0.005
Postintervention period	0.45 (0.26, 0.79)	0.45 (0.24, 0.83)	1.15 (0.56, 2.35)	0.703

[1] All values are geometric $\bar{x}$; 95% CI in parentheses. The probiotic group received *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* (1:1, wt:wt) at a dose of 2–5 × 10^{10} colony-forming units/d.
[2] All results were adjusted for baseline aflatoxin B_1-N^7-guanine (analysis of covariance or ANOVA for repeated measures). Zero values were transformed to the observed minimum value divided by 2, and all values were logarithmically (ln) transformed before the analysis.
[3] ANOVA for repeated measures; $P = 0.003$ for time effect and $P = 0.018$ for interaction between treatment and time.

reported in the current study) could lead to prolongation of the time between initiation and clinical manifestation of cancer from >20 y to >40 y (31). Given that the median age of diagnosis for HCC in many developing countries is <50 y (32), such a delay could have a major effect on health in these and other high-risk areas.

Multiple strategies for the reduction and ultimate prevention of HCC in high-risk populations need to be developed and implemented. Vaccination against HBV is an essential component of the prevention strategy, but even complete elimination of HBV infection would still leave a residual risk of significant magnitude in regions such as China, and it will take more than a generation to achieve overall prevention. Reduction of aflatoxin exposure would therefore be required for effective minimization of the HCC risk. Attempts to reduce dietary aflatoxin exposure quite often requires the commitment of resources to developing technologies for the proper storage and handling of raw food materials to an extent that is not economically feasible at present in many developing countries such as China.

The results of the present probiotic intervention are encouraging for additional studies on an approach of probiotic use that can beneficially influence the toxicokinetics of unavoidable exposures to aflatoxins and other natural and environmental carcinogens. Thus, probiotic-based food products may be an effective dietary prevention approach that could be implemented in many regions of the world to prevent the development of liver cancer or other environmentally induced cancers.

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HSE contributed to the experimental design, analysis and interpretation of the data, and writing of the manuscript. JM, HZ, and WL contributed to the experimental design, running the trial in Guangzhou, and to the analysis and interpretation of dietary questionnaire data. NNP and ROJ contributed to the synthesis of the AFB-N^7-guanine standard and the analysis of urine samples. EKS and SJS contributed to the experimental design and data analysis and provided significant advice in preparing the manuscript. TP contributed to the statistical treatment of the study results. HMM contributed to the experimental design, analysis and interpretation of the data, and critical revision of the manuscript. None of the authors had any conflicts of interest.

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