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Common African cooking processes do not affect the aflatoxin binding efficacy of refined calcium montmorillonite clay

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Abstract

Aflatoxins are common contaminants of staple crops, such as corn and groundnuts, and a significant cause of concern for food safety and public health in developing countries. Aflatoxin B₁ (AFB₁) has been implicated in the etiology of acute and chronic disease in humans and animals, including growth stunting, liver cancer and death. Cost effective and culturally acceptable intervention strategies for the reduction of dietary AFB₁ exposure are of critical need in populations at high risk for aflatoxicosis. Fermented gruels consisting of cornmeal are a common source for such exposure and are consumed by both children and adults in many countries with a history of frequent, high-level aflatoxin exposure. One proposed method to reduce aflatoxins in the diet is to include a selective enterosorbent, Uniform Particle Size NovaSil (UPSN), as a food additive in contaminated foods. For UPSN to be effective in this capacity, it must be stable in complex, acidic mixtures that are often exposed to heat during the process of fermented gruel preparation. Therefore, the objective of the present study was to test the ability of UPSN to sorb aflatoxin while common cooking conditions were applied. The influence of fermentation, heat treatment, acidity, and processing time were investigated with and without UPSN. Analyses were performed using the field-practical Vicam assay with HPLC verification of trends. Our findings demonstrated that UPSN significantly reduced aflatoxin levels (47-100%) in cornmeal, regardless of processing conditions. Upon comparison of each element tested, time appeared to be the primary factor influencing UPSN efficacy. The greatest decreases in AFB₁ were reported in samples allowed to incubate (with or without fermentation) for 72 hrs. This data suggests that addition of UPSN to staple corn ingredients likely to contain aflatoxins would be a sustainable approach to reduce exposure.

Keywords

AFB₁; UPSN; enterosorption therapy; food safety; cornmeal

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1. Introduction

Aflatoxins (AF) are common contaminants of staple crops, such as corn and groundnuts, and are a significant cause of concern for food safety and public health in developing countries. Aflatoxin B₁ (AFB₁) is one of four secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus* and is the most prevalent and toxic of the AF congeners (Wild & Turner, 2002). Frequent humidity, drought, and insect damage in West Africa and South East Asia encourage pre-harvest fungal contamination, leading to chronic AFB₁ exposure in these populations (CAST, 1989). Furthermore, poor grain storage practices that lead to higher moisture levels can cause increased AF levels in harvested grains previously infected in the field (CAST, 2003). Chronic exposure is greatest in communities that produce and consume their own food (Wild & Gong, 2010) and is associated with an increased risk of hepatocellular carcinoma (HCC) (IARC, 1993, 2002; Wild & Turner, 2002). Additionally, AFB₁ is known to be hepatotoxic, genotoxic, immunosuppressive, and anti-nutritional (IARC, 2002).

In many parts of West Africa, populations are chronically exposed to AFs beginning *in utero* (Partanen, et al., 2010; Turner, et al., 2007; Turner, Flannery, Isitt, Ali, & Pestka, 2012). Exposure typically continues through the first years of life, with the presence of a toxic secondary metabolite of AFB₁, Aflatoxin M₁ (AFM₁), in breast milk (Gong, et al., 2003; Shepard, 2008; Zarba, et al., 1992), and well into childhood and adulthood where exposure to AFB₁ can be present in fermented corn meals and porridges (Andah, 1972; Lartey, Manu, Brown, Peerson, & Dewey, 1999). Recently, a sampling of corn-based weaning foods intended for children between the ages of 6 months and 2 years in the Ashanti region of Ghana were found to contain high levels of AFs (Kumi, 2011). All of the 36 samples tested were contaminated with AFs, with 83% containing concentrations above the U.S. FDA action level of 20 ppb AFB₁ and some samples ranging as high as 500 ppb. Despite the fact that fermenting and heating these weaning foods and breakfast gruels may prevent spoilage and enhance food safety, AFs are resistant to degradation by thermal inactivation and fermentation (Christensen, 1977) and therefore remain a constant source of concern.

Methods that focus on reducing dietary exposure to AFs in contaminated foods are highly desirable as a practical strategy to mitigate the harmful effects of this toxin (Williams, et al., 2004). Preferential sorption of AFs in the gastrointestinal tract with the inclusion of certain clays in the diet is one example of this type of approach. NovaSil™ (NS) is a calcium montmorillonite clay with high binding affinity and capacity for AFB₁. NS has been shown to be safe and effective in preventing aflatoxicosis in animals and reducing biomarkers of AF exposure in humans and animals (Harvey, et al., 1991; Kubena, et al., 1991; Lindemann, Blodgett, Kornegay, & Schurig, 1993; Mayura, et al., 1998; Phillips, 1999, 2006; Phillips, Kubena, Harvey, Taylor, & Heidelbaugh, 1988; Phillips, Lemke, & Grant, 2002; Pimpukdee, et al., 2004). These studies have shown that NS is effective as an enterosorbent for AFs when included in the diet at levels ranging from 0.25 to 2% (w/w) in animals (Phillips, et al., 2002). Additionally, a minimal effective dose of NS 0.25% w/w delivered in capsules for three months in a high risk Ghanaian population was successful in decreasing biomarkers of AF exposure and did not interfere with the levels of serum vitamins A and E, iron, or zinc (Afriyie-Gyawu, et al., 2008). Parent NS clay was refined to form UPSN (Uniform Particle Size NovaSil) through a process that served to improve the palatability and consistency of the clay for food delivery. The refining process resulted in a higher percentage of NS particle sizes between 45-100 µm and lower levels of quartz; however, NS and UPSN were compared and shown to have similar AF sorption properties (Marroquin-Cardona, et al., 2011). Rats fed UPSN at levels as high as 2% (w/w) for 13 wk displayed no detectable toxicity (Marroquin-Cardona, et al., 2011). Recently, UPSN inclusion in foods has been investigated in populations at high-risk of AF exposure. In a crossover study in

Ghana, UPSN was shown to be palatable and well-tolerated when added to fermented foods. Moreover, those participants consuming 0.25% UPSN exhibited significantly decreased levels of urinary AFM₁ compared to the placebo group. Also, no adverse reactions from the treatment or placebo were reported. This study indicated that UPSN (when delivered in common fermented foods) was acceptable and could safely and effectively reduce AF exposure when included in contaminated diets (Mitchell, et al., 2013).

Fermentation of corn-based foods in West Africa is common and the effects of acidity and ethanol production during this process are important parameters that could interfere with toxin sorption by clay (UPSN) and thus need to be investigated. Also, knowledge regarding the effects of different cooking conditions (temperature and fermentation time) on the AF-clay complex is needed to determine AF adsorption ability of the clay in a cornmeal matrix. Hence, the objective of the present study was to determine UPSN stability and AFB₁ sorption during fermentation and heating protocols that typify the production of common corn-based foods intended for consumption in this region.

2. Materials and Methods

2.1 Materials

Acetonitrile (ACN) and methanol (MeOH) utilized were HPLC analytical grade (Fisher Scientific, Fair Lawn, NJ). Ultrapure deionized water (18.2 M Ω) was generated using an Ultrapure automated filtration system (Elga™ Woodridge, IL). Aflatoxin B₁ was purchased from Sigma-Aldrich Corporation (St. Louis, Mo). Cornmeal was purchased from a local grocery store in College Station, TX. Extraction equipment, including AflaTest® immunoaffinity columns, was purchased from Vicam® (Watertown, Ma) and utilized according to the manufacturer's instructions. Uniform Particle Size NovaSil (UPSN) was obtained from Texas Enterosorbents (Bastrop, TX). Quantitative analysis of AFB₁ was performed using a Vicam Series 4 Fluorometer and verified on a Waters HPLC with fluorescence detection (Watertown, Ma).

2.2 Cornmeal Preparation

Purchased cornmeal contained an average of 1 ppb AFB₁ as measured by Vicam analysis (see section 2.3). AFB₁ standard was diluted in water to obtain a 25 ppm stock solution for spiking cornmeal samples. The concentration was verified daily using UV spectrophotometry (Shimadzu UV-1800). Cornmeal samples (50 g) were prepared in triplicate, containing 5, 50, 100, 300, 500, and 1000 ppb AFB₁, for both the control and UPSN-treated groups. UPSN (1.5 g) was added to AFB₁-spiked cornmeal samples to comprise the clay test group. The amount of UPSN was calculated based on the high dose of UPSN that was delivered per person in clinical intervention trials in Ghana (1.5 g in each meal) (Mitchell, et al., 2013). AFB₁-spiked samples and clay were mixed together for 15 seconds to disperse the clay throughout the cornmeal. This procedure was repeated for all samples and served as a base mixture before additional processing steps were applied as described in the following Sections 2.2.1, 2.2.2, and 2.2.3.

2.2.1. Base Product—AFB₁ was extracted from samples immediately following the base (non-processed) cornmeal product to assess binding capacity of UPSN for AFB₁ within the cornmeal matrix without any fermentation or cooking. The same procedure was also repeated at pH 3.5 to simulate the average pH observed during the fermentation of corn dough (Plahar & Leung, 1983). This was achieved by adjusting the mixture with HCl until stabilized at pH 3.5.

2.2.2. Fermented Product—Fermentation of the base product was allowed to occur naturally in covered flasks simulating the process used in Africa. Water (50 mL) was added and mixed by agitating the flask until uniform in appearance and thoroughly moist. Mixtures were allowed to ferment for 24 or 72 hr in a NUAIRE™ TS Autoflow Incubator (Plymouth, Mn) maintained at 30° C. This procedure represents both the average temperature and typical fermentation environment that would occur in West Africa. Additionally, samples with the same AFB₁ concentrations and controls were subjected to heat treatment by adding 50 mL boiling water following fermentation. Then, the dough and water were mixed together without further heating, resulting in a matrix with the consistency of a thick soup or gruel. Mixtures were allowed to sit at room temperature for 10 min prior to processing.

2.2.3. Sterilized Product—Sterilized samples were produced by autoclaving the base products and water at 220°C for 30 min prior to the 72 hr incubation period. Throughout this publication, the term “fermentation” will refer to samples that were not sterilized and allowed to ferment by naturally present species of bacteria and other fermenting microorganisms, whereas “sterilized” will refer to samples that were autoclaved and incubated but did not ferment.

2.3 Extraction and Quantification of Aflatoxin

The USDA-FGIS (Federal Grain Inspection Service) single filtration procedure for corn (0-1000 ppb) was used with modifications for the extraction of AFB₁. In brief, 250 mL of water was added to each 50 g cornmeal sample and allowed to mix (2500 rpm) for 30 min on a plate shaker. This step allowed for thorough UPSN interaction with AFB₁ and maintained the clay's interlayer structure responsible for sequestering AFB₁ prior to extraction with a solvent. AFB₁ extraction procedures require use of alcohols and solvents which may disrupt the interlayer structure and decrease AFB₁ binding potential. To circumvent this problem, all samples were first mixed in water to allow for UPSN to bind AFB₁ prior to toxin extraction procedures (Phillips TD, 1990). Following mixing with water, 100 mL of 85:15 acetonitrile:water was added and samples were shaken again for 30 min to extract AFB₁ from the corn matrix. The slurry was then filtered through 8-12 µm × 24 cm fluted filter paper (Vicom). The extract was collected in a clean vessel and filtered a second time through a 1.5 µm × 11 cm glass microfiber filter (Vicom). The final filtered extract was stirred for 10 sec, and 2 mL were passed through an AflaTest immunoaffinity column (Vicom) at a rate of 1-2 drops/second. Columns were washed twice with 3 mL water and eluted with 1 mL MeOH into a glass cuvette. AflaTest developer was added and mixed with the eluate prior to fluorescence detection on a Series 4 Vicam fluorometer with excitation and emission wavelengths of 365 nm and 440 nm, respectively.

To verify overall trends observed with the Vicam analysis, AFB₁ concentrations from representative samples were also measured by HPLC with fluorescence detection. Unlike HPLC, the Vicam method accounts for a loss of AF during immunoaffinity extraction through internal calibrations. AOAC AF extraction protocol (991.31) was utilized for HPLC-fluorescence analysis. For HPLC sample extraction, fermented and sterilized samples spiked with 5, 500, 1000 ppb AFB₁ were prepared as previously mentioned. The samples were then transferred to a blender jar with 250 mL extraction solvent (70:30 MeOH:water) and blended at high speed for 2 min. The slurry was filtered through 8-12 µm × 24 cm fluted filter paper (Vicom) into a clean vessel. The extract was then divided into 10 mL aliquots and diluted with 20 mL water and vortexed. The diluted extract was filtered again through a 1.5 µm × 11 cm glass microfiber filter (Vicom). For samples spiked with 5 ppb AFB₁, 6 mL extract was passed through an AflaTest immunoaffinity column at a rate of 1-2 drops/second. For 500 and 1000 ppb samples, 2 mL extract was passed through the columns. Columns were washed twice with 10 mL water and eluted with 1 mL MeOH into a glass

cuvette. Water (1 mL) was added to each cuvette and mixed well prior to syringe filtering. A mobile phase of 3:1:1 water:ACN:MeOH with a flow rate of 1.0 mL/min was utilized with a Waters Spherisorb C18 column for separation. Corresponding external standards were prepared for each level of AFB₁ and injected (100 µL) prior to sample injection. Concentrations of AFB₁ from 0.1 to 10 µg/mL were linear ($r^2=0.99$) using this method. Excitation and emission wavelengths were set at 360 and 420 nm, respectively. Quantification was achieved by peak-area measurement using Breeze™ HPLC analysis software (Waters). Although different extraction methods were utilized for Vicam and HPLC analysis, comparison of the overall trends between sample groups was the primary focus of this manuscript, rather than comparison of absolute extractable values between AF analytical methods.

2.4 Calculations and Statistical Analysis

All data obtained with the Series 4 Vicam fluorometer was standardized with the values obtained for unfermented cornmeal control samples. For HPLC data, a dilution factor of 5 was applied to samples spiked with 5 ppb AFB₁ and a factor of 15 to the 500 and 1000 ppb AFB₁ samples. JMP 9 software (SAS, Carry, NC) was used to perform factorial analysis of treatments and included Summary of Fit, Analysis of Variance and Student t-test analyses. Percent reduction of AFB₁ was calculated by dividing the treatment average by the control average for each sample concentration. Results were considered significant at $p \leq 0.05$.

3. Results

3.1 UPSN Reduction of AFB₁ in Unfermented Products

Figure 1 demonstrates the binding capacity of UPSN in cornmeal samples at unadjusted (pH 6, Fig. 1a) and acidified pH (pH 3.5, Fig. 1b). A significant decrease in the amount of extractable AFB₁ was observed in the presence of UPSN at both pH values ($P < 0.002$) and percent reductions for unadjusted and pH 3.5 samples ranged from 68-77% and 49-70%, respectively. UPSN-treated sample values were compared between unadjusted and pH 3.5 conditions to assess the effect of acidic pH on the efficacy of UPSN binding. There was no significant difference between 5, 50, 100, 500, and 1000 ppb AFB₁ samples.

3.2 UPSN Reduction of AFB₁ in Fermented Products

In samples that were allowed to ferment for 24 or 72 hr, UPSN significantly reduced the amount of AFB₁ compared to controls (Fig. 2). Percent reduction ranged from 79-88% for 24 hr and from 98-100% for 72 hr fermented products. UPSN binding capacity at 72 hr was enhanced; these samples contained significantly lower AFB₁ levels in the presence of UPSN compared to the 24 hr data at AFB₁ concentrations between 50 to 1000 ppb. Since there was no dose-dependent difference between fermented controls at 24 and 72 hr, these results suggest that AFs were not significantly degraded by fermentation (consistent with the literature), but instead were reduced due to clay sorption processes that were time dependent.

Similarly, samples containing UPSN that were fermented for 72 hr followed by the addition of boiling water (simulating the production of corn-based porridge or gruel in Ghana) significantly reduced AFB₁ levels compared to controls with percent reduction ranging from 91-100% (Fig. 3a). Samples that were treated with UPSN and incubated for 72 hr following sterilization demonstrated significant reduction of AFB₁ in the presence of UPSN ($P < 0.0001$) and total sorption ranged from 85-100% (Fig. 3b).

3.3 HPLC Verification

HPLC fluorometric analysis of sample extracts confirmed UPSN sorption efficacy observed using the Vicam method. The overall trend remained the same between HPLC and Vicam samples. UPSN significantly reduced the amount of AFB₁ present in samples that were unfermented, fermented for 72 hr, or incubated for 72 hr following sterilization (Fig. 4). Percent reductions ranged from 66-92%, 99-100%, and 99-100% for unfermented, fermented, and sterilized samples with UPSN, respectively (Table 1).

4. Discussion

AF contamination in African cornmeal products is a major public health issue that has yet to be resolved; importantly, the vulnerable in susceptible regions are at considerable risk for life-long exposure (IARC, 2012). One suggested solution that has shown promise for wide-scale application is the use of UPSN enterosorption therapy. Since the fermentation of corn-based foods in West Africa is common, the objective of the present study was to determine UPSN stability and AFB₁ sorption during fermentation and heating protocols that typify the production of common corn-based foods intended for consumption in this region. This is a concern because heat treatment via cooking and acid and ethanol created during the fermentation process could potentially interfere with the sorption of AFB₁ on the surfaces of UPSN. Fermentation results in the production of ethanol, which can affect the interlayer stability of calcium montmorillonites such as UPSN. The effects of acidity, fermentation, time of fermentation, and heat application on the ability of UPSN to bind AFB₁ were investigated using a Vicam fluorometer with HPLC validation. By testing cornmeal under these conditions, we were able to determine the difference in UPSN's sorption of AFB₁. In all corn samples, UPSN was able to significantly reduce the amount of extractable AFB₁. This suggests that the clay is stable during fermentation (72 hr) and in the presence of heat while in a food matrix. Thus, the addition of UPSN to foods prior to processing could help to reduce hazardous exposures to AFB₁ from contaminated food sources.

The effect of acidic pH, similar to that produced by the process of fermentation, was assessed and compared to unfermented cornmeal at unadjusted pH. In both conditions, AFB₁ was significantly reduced by UPSN. The lack of significant difference in binding due to alteration of pH is consistent with *in vitro* binding models conducted in our laboratory, where UPSN has previously been shown to be an effective AFB₁ sorbent at a pH as low as 2 from a series of isothermal analyses (Marroquin-Cardona, et al., 2011). This preliminary data supported further work to assess the effects of relevant African food preparation techniques on AF binding by UPSN.

Results from this study indicate that length of fermentation influenced sorption ability, as UPSN was significantly more effective in reducing AF at 72 hr compared to the earlier time points. Our findings suggest that small amounts of UPSN (1.5 g) included in foods, prior to fermentation, could significantly reduce physiologically relevant levels of AFs in corn-based foods. It is possible that this dose delivery platform could result in increased efficacy *in vivo*. When the majority of binding occurs within foods that are hydrated prior to ingestion, the likelihood of potential physiological interactions is reduced, therefore decreasing the risk of exposure and associated toxicities.

Overall, samples that were exposed to heat following fermentation (72 hr) were statistically similar to samples that were fermented without heat. These findings are supported by previous research showing that calcium montmorillonite interlayer structures are stable at temperatures up to 400° C, where the clay can delaminate (Deng Y., 2010) and become inactivated. In order to decrease variability due to time of cooking, the primary preparation step for gruel was simulated in this study. Traditionally, boiling water is added to

reconstitute the matrix and allowed to cook for 2-20 minutes. This boiling time (and the recipe) can vary according to the region and community. Thus, our design was focused on the addition of boiling water to bring the matrix (and AFB₁) to a uniform slurry prior to extraction and analysis. Importantly, we demonstrated that boiling water and/or sterilization temperatures had no effects on the ability of UPSN to bind AFB₁.

Although fermented samples with UPSN exhibited decreased levels of AFB₁, we wanted to determine if treatment time contributed to the effect of UPSN. Sterilized samples were incubated for the same length of time (72 hr) without fermentation. Although comparison between the fermented and sterilized cornmeal did show significantly decreased binding in the sterilized samples with 100 and 500 ppb AFB₁, this trend was not dose-dependent. These findings suggest that the fermentation process itself did not enhance binding, but that time appeared to be the primary factor in the increased sorption of AFB₁ following fermentation. Importantly, inclusion of UPSN in foods before fermentation or cooking does not negatively affect the binding capacity, but allows more opportunity for interaction between UPSN and AFB₁, enhancing its efficacy.

Dietary exposure in Africa is often associated with consumption of both groundnuts and corn; however, it is likely that the majority of AF ingestion occurs through corn-based foods. Exposure assessment and risk characterization for AF-induced health disparities in several African countries indicated that corn consumption, particularly in Ghana, could be as high as 1,000 g per day (Shepard, 2008). Furthermore, dietary intake questionnaires administered by our laboratory in various regions in Ghana revealed that 62% of adults reported consuming corn or corn-based products every day, while only 12% reported daily consumption of groundnuts or groundnut products (Mitchell, 2013 dissertation in proof). However, it is important to note that clays similar to UPSN can effectively bind AF in a peanut matrix (Seifert, et al., 2010), therefore it is likely that UPSN would be effective in a diet consisting of both corn and groundnuts.

Another objective of this study was to develop practical strategies and analytical protocols that would be applicable in parts of the world where AF contamination is often coupled with food scarcity and lack of access to sophisticated analytical technology. Since most field laboratories in areas affected by frequent and high level exposures to AF have the capacity to perform the Vicam assay, we designed this study to use this method. The Vicam assay is relatively inexpensive and field practical which is ideal for situations where rapid screening is desired to monitor and mitigate AF outbreaks. HPLC (with fluorescence detection) was used as a secondary method to validate the ability of UPSN to sorb AF from a food matrix. Although different extraction methods were utilized for Vicam and HPLC analysis, comparison of the overall trends between sample groups was the primary focus of this manuscript, rather than comparison of absolute extractable values between methods. Preliminary work indicated that both methods were similar in their ability to detect the binding of aflatoxins by clay (data not shown).

Furthermore, we hope that this technology can be translated at the village level by including UPSN or similar clays at local mills during corn processing. In the case of AF outbreaks, this would empower the village millers to help prevent aflatoxicosis by adding AF binders to the cornmeal prior to distribution, not unlike iodine inclusion in salt. This novel application could afford villages increased self-sufficiency and entrepreneurial capability. Additionally, advantages of adding the UPSN at the mill include assurance of uniform UPSN distribution within the cornmeal and added consumer convenience.

In this study we were able to demonstrate that a refined calcium montmorillonite clay, UPSN, was able to significantly reduce AFB₁ under common cooking conditions in a corn

meal matrix using a field-practical technique. Therefore, the addition of UPSN to foods at any stage of preparation could be a sustainable approach to alleviate AF-associated public health issues in high-risk populations, enhance the benefits of potentially contaminated foods and nutritional supplements, and empower high risk populations during times of food scarcity and high AF exposures.

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Highlights

- UPSN reduced aflatoxin levels from 47-100% in cornmeal.
- Fermentation, sterilization and heat did not reduce UPSN efficacy.
- Interaction time was the primary factor influencing UPSN efficacy.
- Potential exists for translation of mitigation strategies at the village level.

Figure 1. Reduction of AFB₁ in unfermented, spiked (5-1000 ppb) cornmeal in the presence of UPSN at pH 6 (A) and pH 3.5 (B). Differences between control and UPSN-treated samples were considered significant at *p<0.05. Error bars represent standard error of the mean.

Figure 2.

Reduction of AF in the presence of UPSN after addition of 5-1000 ppb AFB₁ and 24 or 72 hr fermentation. AF reduction between control and UPSN-treated samples at either time point was considered significant at * $p < 0.05$. Significant differences ($p < 0.05$) in AF reduction between 24 and 72 hr are indicated by †. Bars represent standard error of the mean.

Figure 3. Reduction of AFB₁ in the presence of UPSN after heat exposure or sterilization. Cornmeal samples spiked with 5-1000 ppb were allowed to ferment for 72 hr, then exposed to boiling water (A) or sterilized and allowed to incubate without fermentation for 72 hr (B). UPSN reduction of AFB₁ compared to controls (no UPSN) was considered significant at * $p < 0.05$. Bars represent standard error of the mean.

Figure 4. HPLC detection of AFB₁ levels in unfermented, fermented and sterilized samples. All samples were spiked with 1000 ppb AFB₁.

Table 1

Percent AFB1 reduction trends verified by HPLC

Comparison of samples unfermented, fermented for 72 hr, or incubated for 72 hr with and without UPSN.

AFB1 Spiked (ppb)	UPSN (g) ¹	Unfermented 0 hr (ppb) ²	% Reduction ³	Fermented 72 hr (ppb) ²	% Reduction ³	Sterilized & Incubated 72 hr (ppb) ²	% Reduction ³
5	0	18.38		2.78		2.35	
	1.5	6.25	66.0	ND	100.0	ND	100.0
500	0	372.98		254.32		324.11	
	1.5	49.57	86.7	0.77	99.7	4.54	98.6
1000	0	692.41		522.82		617.04	
	1.5	57.11	91.8	57.14	99.2	3.65	99.4

Values were considered significant at $P < 0.05$.

¹Three samples per treatment (n=3).

²AFB1 was recovered at 0 hr (prior to fermentation), at 72 hr with fermentation, and at 72 hr in sterilized samples.

³In all samples containing UPSN, significantly less AFB1 was detected compared to control.