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## Abstract

The effects of fumonisin B-glucose reaction products in swine diets was examined. Pigs were fed diets containing 528  $\mu\text{mol}$  of total fumonisin B/kg (FB), 528  $\mu\text{mol}$  of total FB-glucose adducts/kg (FB-G, 122  $\mu\text{mol}$  of unreacted FB/kg), or 0  $\mu\text{mol}$  of total FB/kg for 15 days to test the efficacy of the FB-G reaction products in detoxifying FB. Weight gain in FB pigs was lower than in FB-G or controls, which was correlated with feed intake reduction in FB pigs. Serum aspartate aminotransferase,  $\gamma$ -glutamyltransferase, and total bilirubin in FB pigs were higher than in FB-G or control pigs. Serum sphinganine/shingosine ratios in FB pigs were higher than in FB-G or control pigs. Microscopic examination of tissues from FB pigs showed generalized liver necrosis and apoptosis with marked cellular pleomorphism and disorganized hepatic cords. The liver and kidneys in the FB-G group appeared to be normal. Tissues of controls were free of lesions. Results suggest that dietary FB-G products are less toxic to swine and may provide an detoxification approach in instances of widespread FB grain contamination ( $p < 0.05$ ).

## Keywords

Interdepartmental Toxicology Program, Department of Veterinary Diagnostic and Production Animal Medicine, Fumonisin B; fumonisin B-glucose; detoxification; swine

## Disciplines

Food Chemistry | Food Science | Human and Clinical Nutrition | Other Animal Sciences

## Comments

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The effects of fumonisin B–glucose reaction products in swine diets was examined. Pigs were fed diets containing 528  $\mu\text{mol}$  of total fumonisin B/kg (FB), 528  $\mu\text{mol}$  of total FB–glucose adducts/kg (FB-G, 122  $\mu\text{mol}$  of unreacted FB/kg), or 0  $\mu\text{mol}$  of total FB/kg for 15 days to test the efficacy of the FB-G reaction products in detoxifying FB. Weight gain in FB pigs was lower than in FB-G or controls, which was correlated with feed intake reduction in FB pigs. Serum aspartate aminotransferase,  $\gamma$ -glutamyltransferase, and total bilirubin in FB pigs were higher than in FB-G or control pigs. Serum sphinganine/shingosine ratios in FB pigs were higher than in FB-G or control pigs. Microscopic examination of tissues from FB pigs showed generalized liver necrosis and apoptosis with marked cellular pleomorphism and disorganized hepatic cords. The liver and kidneys in the FB-G group appeared to be normal. Tissues of controls were free of lesions. Results suggest that dietary FB-G products are less toxic to swine and may provide an detoxification approach in instances of widespread FB grain contamination ( $p < 0.05$ ).

**KEYWORDS:** Fumonisin B; fumonisin B–glucose; detoxification; swine

### INTRODUCTION

Fumonisin (FB) are mycotoxins produced by fungi of the genera *Fusarium*, specifically *F. verticillioides* (= *F. moniliforme*) and *F. proliferatum*, when weather conditions are appropriate for FB production in corn (1). Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is the most common natural contaminant of corn-based foods worldwide (2). It has been associated with a high incidence of esophageal cancer in the Transkei region of South Africa and some provinces of China and was recently declared a class 2B carcinogen or “possibly carcinogenic to humans” (3, 4). In rodents, FB<sub>1</sub> targets the kidneys and liver, where it is capable of inducing cancer (5). Dietary FB<sub>1</sub> levels  $> 1 \mu\text{mol}/\text{kg}$  may be fatal for horses, causing equine leukoencephalomalacia (6, 7). In 1989, a large number of pigs died in the midwestern and southeastern parts of the United States after being fed corn-based diets contaminated with fumonisins from that year’s crop (8). A report by the World Health Organization in 2000 claimed

that globally 59% of corn or samples of corn-based products were contaminated by FB<sub>1</sub> (9).

Swine exposed to subacute dietary levels of 40  $\mu\text{mol}$  of FB/kg for 28 days showed feed intake reduction as well as lower weight gain (10). An increase above normal levels in the serum enzyme activities of liver injury markers such as aspartate aminotransferase (AST),  $\gamma$ -glutamyltransferase (GGT), and the concentration total bilirubin has been reported in swine exposed to FB dietary levels  $> 24 \mu\text{mol}/\text{kg}$  of diet for more than 5 days (11–14). Levels of total cholesterol typically increase significantly in pigs exposed to FB (8). Several studies report that dietary FB levels  $> 139 \mu\text{mol}/\text{kg}$  may be fatal for pigs, causing fatal porcine pulmonary edema (PPE) (8, 11). In swine, FB<sub>1</sub> disrupts sphingolipid metabolism by inhibiting the enzyme ceramide synthase (sphinganine *N*-acetyltransferase) in the de novo sphingolipid synthesis pathway. This leads to accumulation of the sphingoid base sphinganine (Sa) and, to a lesser extent, sphingosine (So). The ratio of Sa to So (Sa/So ratio) may be used as an early biomarker of exposure in pigs fed or administered FB-containing materials (15). Sphinganine concentrations increase in biological samples or tissues such as serum, liver, kidney, heart, or lungs when pigs are exposed to dietary levels in the range of 6  $\mu\text{mol}/\text{kg}$  for 15 days or 10  $\mu\text{mol}/\text{kg}$  for 8 days (15, 16). Morphologic alterations have been

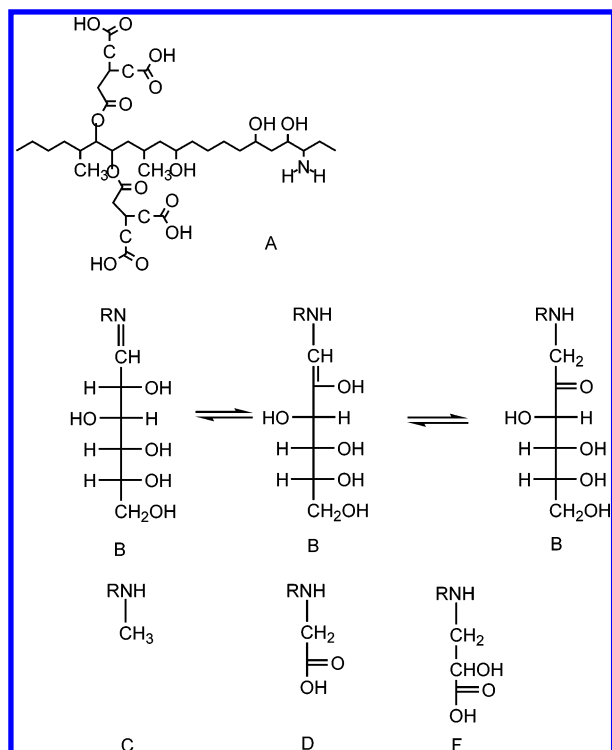
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**Figure 1.** Fumonisin B<sub>1</sub> and identified adducts from reaction with glucose (22): **A**, fumonisin B<sub>1</sub>; R-N = fumonisin B<sub>1</sub> in **B–F**; **B**, [fumonisin B<sub>1</sub>–glucose Schiff base] [or *N*-(deoxy-D-fructos-1-yl)fumonisin B<sub>1</sub>]; **C**, *N*-methylfumonisin B<sub>1</sub>; **D**, *N*-(carboxymethyl)fumonisin B<sub>1</sub>; **E**, *N*-(3-hydroxyacetyl)fumonisin B<sub>1</sub>; **F**, *N*-(2-hydroxy-2-carboxyethyl)fumonisin B<sub>1</sub>.

observed in livers of pigs ingesting diets contaminated with FB<sub>1</sub> at 24  $\mu\text{mol/kg}$  for 5 days. Microscopic lesions consist of random hepatocellular necrosis, nuclear pleomorphism, distorted hepatocytes, hepatomegalocytosis, and increased number of mitotic figures (11, 14).

The primary amine group in the FB<sub>1</sub> molecule has been described as the group required for the toxicity of the molecule (17, 18). Murphy et al. (19) suggested that FB<sub>1</sub> could be detoxified by the formation of a FB<sub>1</sub>-reducing sugar adduct using the nonenzymatic browning (NEB) reaction. Preliminary cell tissue culture tests suggested that these adducts were less toxic than FB<sub>1</sub> (20). This strategy was later used in an animal study where diethylnitrosamine (DEN)-initiated Fischer344/N rats treated with FB<sub>1</sub>–fructose showed a significant reduction in cancer promotion when compared to rats treated with only FB<sub>1</sub> (21). The products of the FB<sub>1</sub>–glucose chain reaction were chemically characterized as *N*-carboxymethylfumonisin B<sub>1</sub> (22, 23), *N*-(1-deoxy-D-fructos-1-yl)fumonisin B<sub>1</sub> (22, 24), *N*-methylfumonisin B<sub>1</sub>, *N*-(3-hydroxyacetyl)fumonisin B<sub>1</sub>, and *N*-(2-hydroxy-2-carboxyethyl)fumonisin B<sub>1</sub> (22) (Figure 1). One of these products, *N*-carboxymethylfumonisin B<sub>1</sub>, was found to be nontoxic in female B6C3F mice fed diets at 140  $\mu\text{mol/kg}$  of diet (25). Promotion of hepatocarcinogenesis and hepatotoxicity was prevented in DEN-initiated female F344/N rats fed diets containing FB<sub>1</sub>–glucose at 35  $\mu\text{mol/kg}$  with 33% unreacted FB<sub>1</sub> (11.1  $\mu\text{mol/kg}$ ) (26). In a more recent study, pigs were injected intraperitoneally with either 5.5  $\mu\text{mol}$  of FB<sub>1</sub>/kg of body weight (BW), 5.5  $\mu\text{mol}$  of FB<sub>1</sub>–glucose products/kg of BW (11% unreacted FB<sub>1</sub> = 0.6  $\mu\text{mol}$  of FB<sub>1</sub>/kg of BW), or 0.9% sterile saline once a day for 7 days. Results of weight gain, liver injury markers, and sphingolipid levels indicated virtually complete liver protection in pigs treated with the FB<sub>1</sub>–glucose reaction products (27).

This study investigates the effects of dietary FB–glucose reaction products in swine using a completely randomized experimental design. The toxicological end point was defined as liver damage. The hypothesis tested was that the chemical reaction of FB with glucose in the diet would decrease the toxicity of FB in swine. The goal was to evaluate the efficacy of the FB–glucose mixture in detoxification of dietary subacute FB poisoning of swine.

## MATERIALS AND METHODS

**Source of Fumonisin B.** *Fusarium moniliforme* M1325 corn culture material containing 9698  $\mu\text{mol}$  of total FB/kg (8590  $\mu\text{mol}$  of FB<sub>1</sub>/kg, 1108  $\mu\text{mol}$  of FB<sub>2</sub>/kg) was obtained from the Veterinary Medical Diagnostic Laboratory at the University of Missouri. The material was appropriately mixed with clean weaning swine feed to obtain the target concentrations of FB in the rations.

**Quantitative Analysis of Fumonisin B According to a Modification of the Method of Murphy et al. (28).** Culture material was ground in a coffee grinder. Five grams was extracted with 100 mL of acetonitrile/water (1:1) for 45 min. After filtering, a 20 mL aliquot was diluted with 30 mL of HPLC grade water and the pH adjusted to 2.7. The extract was centrifuged at 40000g for 15 min. The centrifuged extract was loaded onto a C<sub>18</sub> Sep-pak cartridge (Waters), washed with 5 mL of water/acetonitrile (4:1), and eluted with exactly 2 mL of acetonitrile/water (7:3). Fumonisin B<sub>1</sub> and B<sub>2</sub> were detected as derivatives with *o*-phthalaldehyde following reversed-phase HPLC separation by fluorescence detection (22). One hundred microliters of sample extract was mixed with 100  $\mu\text{L}$  of 50 mM potassium phosphate buffer, pH 8.3, and 100  $\mu\text{L}$  of *o*-phthalaldehyde solution (5 mg of *o*-phthalaldehyde and 10  $\mu\text{L}$  of 2-mercaptoethanol in 5 mL of acetonitrile) at room temperature. After 10 min, the mixture was quenched with 100  $\mu\text{L}$  of water, and the mixture was injected manually into the HPLC system using a 20  $\mu\text{L}$  loop. The *o*-phthalaldehyde solution was stored in the dark at 5  $^{\circ}\text{C}$  and made fresh weekly. The HPLC system included a 250  $\times$  4.6 mm, 5  $\mu\text{m}$  reversed-phase C<sub>18</sub> analytical column (Alltech Associates, Deerfield, IL) and an HPLC fluorescence detector (Waters model 470, Milford, MA) with the excitation wavelength at 335 nm and the emission wavelength at 440 nm. A gradient mobile phase system of 40–60% acetonitrile in 1% aqueous acetic acid at a flow rate of 1.0 mL/min was used. All samples were analyzed in duplicate, and the results were averaged. The minimum detection limit for culture material was 350  $\mu\text{mol}$  of FB<sub>1</sub>/kg of culture material.

**Fumonisin B–Glucose Adduct.** This was prepared following the method of Lu et al. (21) with major modifications. The amount of FB–glucose reaction products needed to obtain 1 kg of feed at 528  $\mu\text{mol}$  of total FB/kg was prepared by adding 28.6 g of 9698  $\mu\text{mol}$  of total FB/kg of *F. moniliforme* M1325 corn culture material (277  $\mu\text{mol}$  of total FB), 9.9 g of 99.7% food-grade dextrose (54939  $\mu\text{mol}$  of glucose) (ADM, Decatur, IL), 4.2 g of food-grade baking soda (49940  $\mu\text{mol}$  of NaHCO<sub>3</sub>), and 200 mL of tap water to a 500 mL autoclavable plastic container. The ratio of FB to glucose was 1:200. The mixture was stirred for 45 min, its pH was checked to be 7.0  $\pm$  0.2, and then the mixture was autoclaved (121  $^{\circ}\text{C}$ , 30 psi) for 90 min. Following heat treatment, the pH was measured again to confirm the buffering activity of NaHCO<sub>3</sub>. The mixture was allowed to cool at room temperature and stored at 4  $^{\circ}\text{C}$  until mixed with 1 kg of weaning feed as previously described. The diet of pigs receiving only FB was prepared by mixing the same amounts of *F. moniliforme* M1325 corn culture material, NaHCO<sub>3</sub>, and tap water but no glucose. In both cases, the final mixtures were HPLC-tested for unreacted FB<sub>1</sub> and FB<sub>2</sub> before and after autoclaving. No differences in FB<sub>1</sub> and FB<sub>2</sub> concentrations were observed between before and after autoclaving culture material when glucose was not added.

**Animals.** The use of animals and experimental procedures were approved by the Iowa State University Laboratory Animal Care Committee in 2003. Eighteen 3-week-old crossbred healthy pigs (mean starting body weight = 6.3 kg  $\pm$  0.08) were housed in individual pens (width = 1.23 m, length = 1.23 m, height = 0.76 m) and allowed to acclimate to the animal room for 3 days. Room temperature was maintained at 27  $^{\circ}\text{C}$ , and water and feed were supplied ad libitum.

**Treatments.** Pigs were randomly divided into three treatment groups of six pigs each. Treatments were fumonisin B (FB), fumonisin–glucose (FB-G), and control. All pigs were daily fed 25% more feed than their typical intake to ensure enough feed availability. FB pigs were fed 528  $\mu\text{mol}$  of total FB/kg of diet (342  $\mu\text{mol}$  of FB<sub>1</sub>/kg diet, 186  $\mu\text{mol}$  of FB<sub>2</sub>/kg of diet). Pigs in the FB-G group were fed a diet containing nominally 528  $\mu\text{mol}$  of total FB/kg of diet with ~23% glucose-unreacted FB (122  $\mu\text{mol}$  of total FB/kg of diet). Levels of FB<sub>1</sub> and FB<sub>2</sub> were 79  $\mu\text{mol}$  of FB<sub>1</sub>/kg of diet and 43  $\mu\text{mol}$  of FB<sub>2</sub>/kg of diet, respectively. Control pigs were fed diets with no detectable FB (limit of detection = 0.4  $\mu\text{mol}$  of total FB/kg of diet). On the basis of the levels of dietary FB exposure and feed intake data, pigs in the control group received a daily average oral FB dosage of 0  $\mu\text{mol}$ /kg of BW, whereas FB and FB-G pigs received dosages of 21.6 and 8.9  $\mu\text{mol}$ /kg of BW, respectively. All rations contained 20% moisture.

**Diets.** All pigs received a balanced diet consisting of weaning feed (Nevada Feed and Seed Co., Nevada, IA) with the following approximate composition: dry matter, 88.8%; protein, 18.76%; lysine, 1.35%; fat, 4.74%; fiber, 3.22%; calcium, 0.91%; phosphorus, 0.75%; salt, 0.55%; zinc, 139.4 ppm; iodine, 0.02 ppm; iron, 19.8 ppm; copper, 0.9 ppm; manganese, 3.42 ppm; selenium, 0.28 ppm; and vitamin E, 3.96 IU/kg. This feed was the basis of the diet for all pigs in the study.

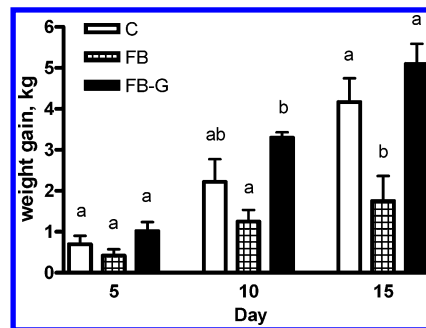
**General Health.** The pigs were evaluated using a checklist form specifically designed to record simple clinical observation parameters including behavior, mucous membrane coloration, respiratory rate (resp/min), heart rate (beats/min), and rectal temperature ( $^{\circ}\text{C}$ ) on a daily basis. Weight gain was calculated on day 15 of the study by subtracting initial (day 0) from final body weight (day 15).

**Clinical Chemistry.** Pigs were bled from the orbital venous sinus on days 0, 5, 10, and 15. Blood was allowed to clot for at least 45 min and then centrifuged for 15 min to separate serum from the clot (International Equipment Co., Needham Heights, MA). All serum samples were evaluated for liver enzymes AST and GGT and total bilirubin (BIL) and total cholesterol (CHOL) using a Hitachi 912 programmable, automated clinical analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Duplicate serum samples were stored at  $-20^{\circ}\text{C}$  for extraction and HPLC analysis of sphingolipids.

**Liver Total Cholesterol.** All samples were analyzed in triplicates. Ten grams of liver was homogenized in 30 mL of 100% HPLC-grade methanol. Six milliliters of 90% potassium hydroxide was added to a 3.0 mL aliquot of the homogenate to hydrolyze the fatty acid ester linkages. Samples were refluxed for 30 min under a water-cooled condenser. The mixture was allowed to cool, 15 mL of distilled water was added, and the solution was transferred to a separatory funnel. An equal volume (24 mL) of 100% HPLC-grade hexane was added. The aqueous layer was washed with hexane three times. The three hexane fractions were pooled and evaporated to dryness in a rotary evaporator. The cholesterol residue was redissolved in 1 mL of 100% ethanol by stirring and/or sonication and analyzed or stored at  $4^{\circ}\text{C}$  until the next day. Samples were analyzed enzymatically using cholesterol oxidase (100 units/L), peroxidase (800 units/L), 0.25 mM 4-aminoantipyrine, 10 mM hydroxybenzoic acid (Sigma, St. Louis, MO), 50 mM potassium phosphate buffer (Fisher Scientific, Pittsburgh, PA), and 0.1% sodium azide (Eastman Kodak, Rochester, NY). A 30  $\mu\text{L}$  aliquot of the redissolved samples in ethanol solution was mixed with 1.5 mL of the enzyme mixture, stirred for 30 s, and incubated at room temperature for 1 h. The absorbance of samples was read in a DU 520 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 550 nm. A bovine brain cholesterol standard (Matreya, Inc., Pleasant Gap, PA) dissolved in ethanol was used to prepare standard curves.

**Sphingolipid Analysis.** The concentrations of Sa and So and their ratio were measured in serum and liver. Extraction, internal standard curve, and HPLC analysis were performed as in Fernández-Surumay et al. (27). Thirty microliters of 5  $\mu\text{M}$  *d,l*-erythro-C20-dihydrospingosine (C-20, 50 pmol) (Matreya, Inc.) internal standard was added to the samples during extraction.

**Pathological Evaluation.** All pigs were necropsied on day 15 after euthanasia with a lethal intravenous dose of 0.5 mL of a 26% sodium pentobarbital solution. Weights of potential target organs liver, heart, and kidneys were obtained and compared to final body weight to construct organ weight/body weight ratios. Tissue samples from liver



**Figure 2.** Weight gain comparison between pigs fed 0 (control), 528 (FB), and 528  $\mu\text{mol}$  of total FB/kg of diet with 23% unreacted FB (122  $\mu\text{mol}$  of total FB/kg of diet) (FB-G).  $n = 6$  pigs. The error bar represents the standard error of the mean (SEM). Different letters above error bars indicate statistically significant differences between the treatment groups at a given time point ( $p < 0.05$ ). Average initial body weights (day 0) did not differ significantly among the groups.

and kidneys were fixed in 10% neutral buffered formalin, processed routinely, sectioned at 5  $\mu\text{m}$ , and stained with hematoxylin and eosin for histological evaluation. Additional liver tissue samples were stored at  $-20^{\circ}\text{C}$  for further sphingolipid extraction.

**Statistical Analysis.** All tests were performed using SAS software v. 8.02 (SAS Institute Inc., Cary, NC, 2001). For each of the response variables AST, GGT, BIL, CHOL, serum Sa, serum So, serum Sa/So ratio, weight gain, and feed intake, within-day treatment comparisons of the least-squares means were made by a repeated-measurements analysis using the MIXED procedure. For each response variable, the variance component of the RANDOM effects parameter (experimental subject nested in treatment) was estimated by method of moments. A compound symmetric covariance structure was assumed for each subject. Mean comparisons of response variables liver Sa, liver So, liver Sa/So ratio, and liver CHOL were adjusted with the Tukey method for multiple comparisons. A  $P$  value of 0.05 was considered to be significant except where noted.

## RESULTS

We proposed that dietary FB–glucose reaction products would decrease liver toxicity in pigs fed subacute dietary levels of FB. In pigs receiving 528  $\mu\text{mol}$  of FB/kg of diet, the clinical signs were mild to moderate and became obvious at day 4. These signs included weakness, rough hair, and lateral recumbency. Icterus in skin of the ventral abdominal and inguinal region was observed from day 10 to day 15. The FB-G pigs, consuming 23% unreacted FB of a 528  $\mu\text{mol}$  of FB/kg diet (122  $\mu\text{mol}$  of FB/kg of diet) showed no apparent signs of disease, except for mild diarrhea in one pig on days 1 and 2. The control pigs (<0.4  $\mu\text{mol}$  of FB/kg of diet) did not show any apparent signs of disease throughout the duration of the study. No pigs died during the study. The total average feed intake as a percentage of body weight was statistically similar among controls ( $6 \pm 0.6\%$ ) and FB-G ( $6.2 \pm 0.4\%$ ) pigs. Both groups had a higher feed intake than FB pigs ( $3.8 \pm 0.3\%$ ). As a result, FB pigs at day 15 had a significantly lower total weight gain ( $1.75 \pm 0.61$  kg) as compared to controls ( $4.17 \pm 0.58$  kg) and FB-G ( $5.1 \pm 0.49$  kg) pigs, which were not different from each other ( $p < 0.05$ ) (Figure 2).

On day 0 of the study, serum enzyme activities of AST and GGT and levels of total cholesterol were similar among the treatment groups (Table 1). Although the animals were randomly assigned to the experimental groups, total bilirubin was higher in controls as compared to FB-G pigs. In FB pigs, total bilirubin was similar to control and FB-G pigs. These differences were not related to the treatments and could have been due to stress events previous to FB exposure in the study. On day 5,

**Table 1.** Enzyme Activities of Aspartate Aminotransferase (AST) and  $\gamma$ -Glutamyl Transferase (GGT) and Levels of Total Bilirubin (BIL) and Total Cholesterol (CHOL) in Serum on Days 0, 5, 10, and 15 of the Study (FB Levels in Diets: 528  $\mu$ mol/kg in FB Group, 122  $\mu$ mol/kg in FB-Glucose Group)

	AST (IU/L)	GGT (IU/L)	BIL (mg/dL)	CHOL (mg/dL)
day 0				
control	34.8 $\pm$ 3.9 a	28.5 $\pm$ 5.5 a	1.8 $\pm$ 0.5 a	257.7 $\pm$ 15.3 a
FB	33 $\pm$ 4.0 a	31.3 $\pm$ 5.2 a	1.1 $\pm$ 0.4 ab	235.5 $\pm$ 32.1 a
FB-G	30.3 $\pm$ 2.2 a	41.7 $\pm$ 5.1 a	0.5 $\pm$ 0.2 b	293.5 $\pm$ 24.4 a
day 5				
control	32.3 $\pm$ 3.8 b	30.0 $\pm$ 4.4 b	0.1 $\pm$ 0.0 b	129 $\pm$ 25.3 a
FB	212.8 $\pm$ 42.6 a	65.7 $\pm$ 7.6 a	1.5 $\pm$ 0.5 a	166.2 $\pm$ 18.1 a
FB-G	54.3 $\pm$ 12.3 b	50.2 $\pm$ 9.4 ab	0.1 $\pm$ 0.0 b	83.7 $\pm$ 5.9 b
day 10				
control	36 $\pm$ 3.8 b	29.2 $\pm$ 4.6 b	0.1 $\pm$ 0.0 b	68.0 $\pm$ 3.1 b
FB	414.3 $\pm$ 56.9 a	189 $\pm$ 43 a	2.9 $\pm$ 0.8 a	196.2 $\pm$ 40.2 a
FB-G	40.8 $\pm$ 2.9 b	50.0 $\pm$ 7.0 b	0.1 $\pm$ 0.0 b	89.3 $\pm$ 4.4 b
day 15				
control	120.2 $\pm$ 18.7 c	47.3 $\pm$ 10.1 c	0.1 $\pm$ 0.0 b	76.3 $\pm$ 4.3 b
FB	576 $\pm$ 83 a	251 $\pm$ 48 a	3.3 $\pm$ 1.2 a	204.8 $\pm$ 32.4 a
FB-G	180.7 $\pm$ 51.1 b	99.3 $\pm$ 23.3 b	0.4 $\pm$ 0.3 b	119.2 $\pm$ 29.3 b

<sup>a</sup> The data entries in the table are expressed as mean  $\pm$  SEM ( $n = 6$  pigs). Different letters indicate statistically significant differences between the group means at a given time point ( $p < 0.05$ ).

FB pigs showed moderately elevated AST and total bilirubin with respect to controls. FB pigs had GGT levels higher than those of controls and similar to those of FB-G pigs, although not significantly elevated. Serum cholesterol was higher in FB and control pigs when compared to FB-G pigs, but not significantly elevated. On day 10, AST and total bilirubin were markedly elevated in FB pigs as compared to controls and FB-G pigs, which were not different. Levels of GGT and total cholesterol were also higher in FB pigs but to a lesser extent. On day 15, all AST, GGT, total bilirubin, and total cholesterol showed markedly elevated levels in FB pigs as compared to controls and FB-G pigs, making the difference between FB pigs and controls and FB-G pigs even more pronounced ( $p < 0.05$ ). The total liver cholesterol levels in FB pigs ( $4.5 \pm 0.62 \mu$ mol of total cholesterol/g of liver) were significantly higher than in FB-G ( $1.95 \pm 0.65 \mu$ mol of total cholesterol/g of liver) ( $p < 0.05$ ) and control pigs ( $2.64 \pm 0.67 \mu$ mol of total cholesterol/g of liver) ( $p < 0.1$ ). Levels of controls and FB-G pigs were not different from each other.

**Table 2.** Levels of Sphinganine (Sa) and Sphingosine (So) and the Sa/So Ratio in Serum on Days 0, 5, 10, and 15 of the Study (FB Levels in Diets: 528  $\mu$ mol/kg in FB Group, 122  $\mu$ mol/kg in FB-Glucose Group)<sup>a</sup>

	Sa (nmol/mL)	So (nmol/mL)	Sa/So
day 0			
control	0.4 $\pm$ 0.14 a* ( $n = 14$ )	1 $\pm$ 0.27 a ( $n = 11$ )	0.44 $\pm$ 0.15 a ( $n = 10$ )
FB	0.3 $\pm$ 0.15 ab* ( $n = 16$ )	0.75 $\pm$ 0.33 a ( $n = 17$ )	0.49 $\pm$ 0.15 a* ( $n = 15$ )
FB-G	0.1 $\pm$ 0.02 b ( $n = 16$ )	0.48 $\pm$ 0.08 a ( $n = 16$ )	0.26 $\pm$ 0.06 a* ( $n = 16$ )
day 5			
control	0.12 $\pm$ 0.02 b* ( $n = 18$ )	0.56 $\pm$ 0.08 a ( $n = 17$ )	0.3 $\pm$ 0.07 b* ( $n = 17$ )
FB	0.6 $\pm$ 0.08 a ( $n = 17$ )	0.7 $\pm$ 0.19 a ( $n = 18$ )	1.3 $\pm$ 0.19 a ( $n = 17$ )
FB-G	0.16 $\pm$ 0.02 b* ( $n = 19$ )	0.47 $\pm$ 0.09 a ( $n = 18$ )	0.52 $\pm$ 0.1 b* ( $n = 18$ )
day 10			
control	0.09 $\pm$ 0.01 c ( $n = 17$ )	0.47 $\pm$ 0.16 b ( $n = 16$ )	0.6 $\pm$ 0.15 b ( $n = 15$ )
FB	0.76 $\pm$ 0.13 a ( $n = 18$ )	0.61 $\pm$ 0.12 a* ( $n = 16$ )	1.83 $\pm$ 0.34 a* ( $n = 16$ )
FB-G	0.28 $\pm$ 0.09 b ( $n = 16$ )	0.53 $\pm$ 0.15 a* ( $n = 14$ )	0.95 $\pm$ 0.16 a* ( $n = 10$ )
day 15			
control	0.21 $\pm$ 0.05 c ( $n = 18$ )	0.53 $\pm$ 0.14 a* ( $n = 18$ )	0.5 $\pm$ 0.08 c ( $n = 16$ )
FB	0.92 $\pm$ 0.06 a ( $n = 19$ )	0.64 $\pm$ 0.09 a* ( $n = 18$ )	1.78 $\pm$ 0.18 a ( $n = 18$ )
FB-G	0.5 $\pm$ 0.07 b ( $n = 18$ )	0.78 $\pm$ 0.25 a ( $n = 18$ )	1.14 $\pm$ 0.2 b ( $n = 16$ )

<sup>a</sup> The concentration of sphingolipids is expressed as nanomoles per milliliter of serum. The data entries in the table are expressed as mean  $\pm$  SEM. Different letters indicate statistically significant differences between the group means at a given time point ( $p < 0.05$ ). \*, significantly different at  $p < 0.1$ .  $n =$  repeated extractions.

The serum levels of Sa in FB pigs on day 0 were similar to those of control and FB-G groups, whereas FB-G pigs showed Sa levels lower than in controls (**Table 2**). Serum levels of So and the Sa/So ratio were not different between the treatment groups. On day 5, Sa levels in FB pigs were higher than in control and FB-G pigs. Levels of So were similar between the treatment groups. Similar to Sa, the Sa/So ratio in the FB group was higher than in controls and FB-G pigs. On day 10, Sa levels were highest in FB pigs followed by FB-G and controls. The concentration of So was higher in FB and FB-G groups as compared to controls. The Sa/So ratio followed the same pattern as So levels. On day 15, Sa levels continued to be highest in the FB group followed by FB-G and control pigs. Levels of So were similar between the treatment groups, whereas the Sa/So ratio, as Sa levels, was highest in the FB group followed by FB-G and control pigs ( $p < 0.05$ ). In contrast, liver levels of Sa and So were highest in FB pigs at necropsy followed by FB-G and controls (**Table 3**). The liver Sa/So ratio in FB pigs was higher than in controls but not different from the FB-G group. The liver ratios for control and FB-G pigs were not different from each other ( $p < 0.05$ ).

On gross pathology inspection, five of six FB pigs showed icterus of subcutaneous, abdominal, and pericardial fat. In one FB pig, yellow discoloration of these areas was not as severe. Livers of all FB pigs, although not enlarged, showed yellowish tan irregular areas indicative of abnormal accumulation of bilirubin. Lungs had a normal diffuse pink color, were not edematous, and had clear airways and trachea. Kidneys appeared to be normal on external and cut surfaces. Serosal surfaces of the gastrointestinal tract appeared to be mildly congested but not hemorrhagic. Two of six FB-G pigs showed livers with pale yellowish surface areas that did not appear to be irregular. The rest of the FB-G pigs and all control pigs had no obvious signs of icterus and were apparently free of lesions. Microscopic liver changes in FB pigs consisted of individual hepatocyte necrosis, shrunken hepatocytes with condensed chromatin indicative of apoptosis, disorganized hepatic cords, and marked cellular pleomorphism with megalocytosis. No microscopic lesions were observed in kidneys of FB-G or control pigs. Livers in the FB-G group appeared to be normal except for mild individual hepatocyte necrosis and disorganization of hepatic cords in the two FB-G pigs that showed pale yellowish surface areas on gross evaluation. Tissues of control pigs were free of

**Table 3.** Liver Levels of Sphinganine (Sa) and Sphingosine (So) and the Sa/So Ratio in Liver on Day 15 of the Subacute Study (FB Levels in Diets: 528  $\mu\text{mol/kg}$  in FB Group, 122  $\mu\text{mol/kg}$  in FB-Glucose Group)<sup>a</sup>

	Sa (nmol/g)	So (nmol/g)	Sa/So
control	0.3 $\pm$ 0.03 <b>c</b> ( <i>n</i> = 18)	20.9 $\pm$ 6.4 <b>c</b> ( <i>n</i> = 13)	0.03 $\pm$ 0.04 <b>b</b> ( <i>n</i> = 13)
FB	6.2 $\pm$ 0.4 <b>a</b> ( <i>n</i> = 18)	69.4 $\pm$ 9.9 <b>a</b> ( <i>n</i> = 17)	0.15 $\pm$ 0.03 <b>a</b> ( <i>n</i> = 17)
FB-G	3.02 $\pm$ 0.4 <b>b</b> ( <i>n</i> = 18)	48.6 $\pm$ 9.4 <b>b</b> ( <i>n</i> = 18)	0.11 $\pm$ 0.03 <b>ab</b> ( <i>n</i> = 18)

<sup>a</sup> The concentration of sphingolipids is expressed as nanomoles per gram of liver tissue. The data entries in the table are expressed as mean  $\pm$  SEM. Different letters indicate statistically significant differences between the group means (*p* < 0.05). Levels of So in the FB group were different from those in the FB-G group at a 10% significance level. *n* = repeated extractions.

lesions. A comparison of the three treatment groups is presented in **Figure 3**. Ratios of organ weight to total body weight for heart, lungs, liver, or kidney showed no significant differences between the treatment groups (data not shown).

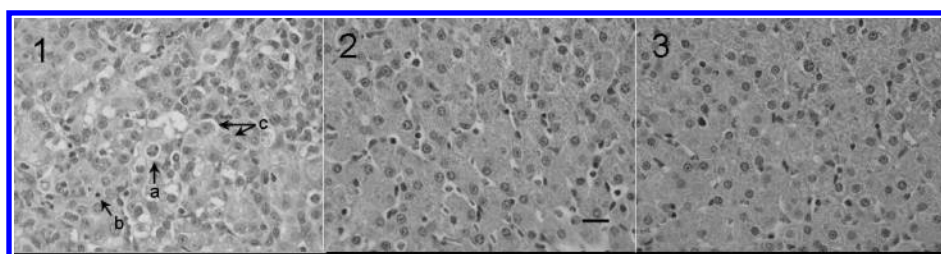
## DISCUSSION

In preparation for the present study, two preliminary dietary trials on the detoxifying effects of the FB–glucose reaction products in swine were performed. The goal of the first study was to determine the efficacy of these products on protecting pigs fed 97  $\mu\text{mol}$  of total FB/kg of diet with 19% unreacted FB (18  $\mu\text{mol}$  of total FB/kg of diet) for a 25 day period against liver damage. Controls and FB pigs were fed 0 and 97  $\mu\text{mol}$  of total FB/kg of diet, respectively, with three pigs in each group. We chose to feed 97  $\mu\text{mol}$  of total FB/kg of diet on the basis of previous studies in the literature using culture material or naturally contaminated corn screenings in which liver damage was the end point (11–14). Our serum chemistry results indicated that a 97  $\mu\text{mol}$  of total FB/kg of diet level for a 25 day period was not sufficient to cause clinically recognizable liver damage in our FB pigs. The enzyme activities of liver injury markers, AST (132 IU/L  $\pm$  27) and GGT (58 IU/L  $\pm$  8), and the levels of total bilirubin (0.2 mg/dL  $\pm$  0.04) at the end of the study were either within normal levels (29) or <1-fold higher than the upper normal limit. Because we were not able to induce liver damage at these FB levels in these pigs, we could not demonstrate protection from the FB–glucose reaction products. To find the appropriate dietary FB level to cause clinically recognizable liver damage, we designed a new pilot study in which four pigs were fed 396 and 528  $\mu\text{mol}$  of total FB/kg of diet (two animals per FB level) for a 16 day period. Decreased body weight gains and highly elevated or above normal range enzyme activities of AST (557 IU/L) and GGT (185 IU/L) and levels of total bilirubin (3.48 mg/dL) in pigs fed 528  $\mu\text{mol}$  of total FB/kg of diet indicated severe liver

damage. Hence, a total FB dietary level of 528  $\mu\text{mol/kg}$  of diet was selected for the study subject of this paper.

In general, the data collected in the present study revealed that diets containing FB–glucose reaction products at a concentration of 528  $\mu\text{mol}$  of total FB/kg of diet with  $\sim$ 23% unreacted FB (122  $\mu\text{mol}$  of total FB/kg of diet) dramatically diminished liver damage. Protection of FB-sensitive species by FB–fructose or FB–glucose adducts has been previously reported in rat (21, 26), mouse (25), and swine (27) models. In the present study, total weight gain of FB pigs was significantly lower than that of FB-G or control pigs as a result of decreased feed intake. Significantly lower weight gains have been reported in swine exposed to diets containing 33  $\mu\text{mol}$  of total FB/kg for 14 days (10, 14). Heat treatment of FB-contaminated feeds with reducing sugars such as glucose will prevent the weight loss problem observed in swine.

The serum enzyme activities of liver injury markers AST, GGT, and total bilirubin in pigs fed 528  $\mu\text{mol}$  of total FB/kg of diet with 100% unreacted FB were elevated above the normal range (29) and were significantly higher than in pigs receiving FB–glucose products or control feed on days 10 and 15 of the study. The levels of these markers have been previously reported to increase above normal levels in pigs exposed to dietary FB levels >24  $\mu\text{mol}$  of total fumonisin B/kg of diet for more than 5 days (11–14). On day 15, however, FB-G pigs showed AST and GGT levels above the normal range and significantly higher than those in controls but still lower than those in the FB group. The difference between FB-G and control groups could be explained by the total unreacted-FB level in the diet of pigs in the FB-G group (122  $\mu\text{mol}$  of total FB/kg), which was considerably higher than in controls (0  $\mu\text{mol}$  of total FB/kg). An alternative explanation is that the dietary FB–glucose reaction products in the diet of FB-G pigs could have induced mild liver damage characterized by a modest increase of AST and GGT enzyme activities. The FB–glucose products fed to the swine in this study have not been chemically characterized. A study by Lu et al. (22) characterized the kinetics and products of the FB<sub>1</sub>–glucose NEB reaction in a corn model system. One of the characterized products, *N*-carboxymethylfumonisin B<sub>1</sub>, was fed by Howard et al. (23) to female B6C3F mice at 14, 70, and 140  $\mu\text{mol/kg}$  of diet. Results indicated that this FB derivative did not alter serum analytes, organ weights, or hepatic structure in the mice. As we might hypothesize that one or more of the characterized FB–glucose reaction products could have been present in the diet of our FB-G pigs, their individual toxicity remains unknown and should be the object of future study. Levels of total cholesterol in serum followed the same pattern of total bilirubin. These were significantly higher and elevated above normal levels in FB pigs as compared to FB-G or control pigs. These results agree with Haschek et al. (8), who



**Figure 3.** (A) Liver from a fumonisin B-treated pig with an individual necrotic cell (a), a shrunken hepatocyte with condensed chromatin (apoptosis) (b), disorganized hepatic cords, and marked cellular pleomorphism with megalocytosis (c). (B) Liver from a pig in the control group. Significant microscopic lesions were not observed. (C) Liver from a fumonisin B–glucose-treated pig. Significant microscopic lesions were not observed in four of six pigs. Hematoxylin and eosin stain. Bar = 30  $\mu\text{m}$ .

described hypercholesterolemia as one of the most sensitive serum biochemical parameters of fumonisin exposure.

The statistical differences in liver total cholesterol between the treatment groups were similar to those observed in the analysis of serum total cholesterol. Contrary to what we expected, liver total cholesterol was higher in FB pigs as compared to FB-G and control pigs. The enzyme hydroxymethylglutaryl-CoA reductase is described as the primary control point for cholesterol synthesis in the hepatocyte. Artificially increasing plasma cholesterol levels *in vivo* decreased the activity of the enzyme in liver by repressing the synthesis of the enzyme mRNA (30). This is consistent with Julius et al. (31), who reported in their study of infant formulas and their effect on weanling pigs that liver cholesterol concentrations were inversely related to concentrations in plasma. However, the inhibition of the enzyme ceramide synthase in the pigs fed FB in this study could have affected the cholesterol metabolism in the liver. The bulk of cellular free cholesterol and sphingomyelin, one of the major sphingolipids and levels of which decrease after ceramide synthase inhibition, interact strongly with each other through hydrogen bonding and van der Waals interactions to form cholesterol/sphingolipid-enriched domains or so-called "lipid membrane rafts" in the plasma and other organelle membranes (32, 33). The interactions between sphingomyelin in the intracellular transport and metabolism of cholesterol would not be unexpected (34). We hypothesize that the inhibition of ceramide synthase in our FB pigs ultimately led to a decrease in the synthesis of cell membrane sphingomyelin, which could have activated the synthesis of cholesterol esters in the liver. This is supported by Chatterjee (35), who found an increase in cholesterol esters after reducing the levels of sphingomyelin by incubating human skin fibroblasts with the bacterial enzyme neutral sphingomyelinase. Moreover, in the present study the levels of sphingomyelin in the pigs fed FB could have been reduced even further by an increase in the levels of tumor necrosis  $\alpha$  (TNF $\alpha$ ). This cytokine has been found to increase the activity of sphingomyelinase in cultured human fibroblasts, and interestingly, has also been found in increased levels in macrophages exposed to FB<sub>1</sub> (35, 36). The possible complex interactions, if any, between FB, TNF $\alpha$ , sphingolipid, and cholesterol metabolism in the liver remain to be studied.

It is well-known that FB disrupts sphingolipid metabolism in swine. The results of serum Sa and Sa/So ratios indicated protection of the FB-G pigs. Blocking of the primary amine groups of FB<sub>1</sub> and FB<sub>2</sub> by their reaction with glucose likely prevented the inhibition of the enzyme ceramide synthase in the *de novo* sphingolipid synthesis pathway. Therefore, a significant accumulation of sphingolipid precursors Sa and So was not observed in the FB-G group. However, protection was not as evident as in our earlier study (27). In that study, pigs exposed to FB<sub>1</sub>-glucose reaction products at 5.5  $\mu\text{mol/kg}$  of BW/day (= 0.6  $\mu\text{mol}$  of FB<sub>1</sub>/kg of BW/day) by intraperitoneal injection for 7 days showed significantly lower serum and liver levels of Sa and a lower Sa/So ratio than pigs administered FB<sub>1</sub> at 5.5  $\mu\text{mol/kg}$  of BW/day or control pigs. In the present study, pigs fed FB-glucose reaction products at 528  $\mu\text{mol}$  of FB/kg of diet had serum Sa levels and Sa/So ratios significantly lower than pigs fed FB at 528  $\mu\text{mol}$  of FB/kg of diet but higher than control pigs at day 15. As in the case of liver injury markers AST and GGT, this could be explained by the difference in total FB dietary levels between these two groups. In the present study, FB pigs received an average oral FB dosage of 21.6  $\mu\text{mol/kg}$  of BW/day, whereas FB-G pigs received 8.9  $\mu\text{mol/kg}$  of BW/

day. According to Prelusky et al. (37), the oral bioavailability of FB<sub>1</sub> in swine is  $\sim 4.5\%$ . If we apply this proportion to the average FB oral dosages in our study, FB and FB-G pigs absorbed an average of 1 and 0.4  $\mu\text{mol}$  of FB/kg of BW/day, respectively. Considering only average daily FB dosages, in Fernández-Surumay et al. (27) a difference of 0.6  $\mu\text{mol}$  in the FB<sub>1</sub> dosage of FB<sub>1</sub>-G and control pigs was not sufficient to elicit different Sa level and Sa/So ratio responses in these two groups. In contrast, a difference of 0.4  $\mu\text{mol}$  between the same groups in the present study caused higher Sa levels and Sa/So ratios in FB-G pigs as compared to controls, possibly caused by a higher susceptibility to FB in the pigs used in this study. In general, a significant increase in the Sa/So ratio has been previously reported in pigs consuming diets with FB<sub>1</sub> concentrations of 13.9  $\mu\text{mol/kg}$  of diet for only 8 days (16, 38). An increase in the concentration of Sa and the Sa/So ratio has also been observed in pigs fed 32  $\mu\text{mol}$  of FB<sub>1</sub>/kg of diet (15). In the present study, concentrations of Sa as well as the Sa/So ratio showed a time-dependent elevation in the FB and FB-G groups, which agrees with studies by Smith et al. (39) and Gumprecht et al. (40). The levels of So were not different among the three groups throughout the majority of the present study (days 0, 5, and 15). The difference in So levels observed on day 10 between controls and FB and FB-G pigs was considered to be temporal as it disappeared on day 15. As we mentioned in the case of serum AST and GGT, another explanation for the difference in Sa levels and the Sa/So ratio between control and FB-G pigs could be that the dietary FB-glucose reaction products might have inhibited ceramide synthase, causing elevation of Sa and the Sa/So ratio in this group. Interactions between specific FB-glucose reaction products and the enzyme ceramide synthase in the sphingolipid biosynthesis pathway deserve further investigation. Liver levels of Sa and So in FB-exposed pigs were significantly higher than in pigs fed FB-glucose products or controls, which was indicative of protection by the FB-glucose products. This agrees with Fernández-Surumay et al. (27), who reported a protective effect in Sa levels by FB-glucose products. Significant elevations of free Sa and So in liver have been previously reported in pigs fed diets containing 32  $\mu\text{mol}$  of FB/kg of diet for 14 days (15). As in the case of serum Sa on day 15, liver Sa and So were higher in FB-G pigs as compared to controls. As we mentioned before, this could be due to the FB level in the diet of FB-G pigs, inhibition of ceramide synthase by FB-glucose products, or both. In contrast to the findings of Fernández-Surumay et al. (27), the liver Sa/So ratio in FB-G pigs, although lower than in FB pigs, was not significantly different from either FB or control groups. The higher values of Sa/So ratios observed in livers of FB-G pigs were likely a product of their high Sa liver levels as the ratio is a direct relationship between Sa and So levels (41). In general, the level of sphinganine appeared to be a more sensitive biomarker of exposure to FB than the Sa/So ratio in the present study. Liu et al. (26) reached the same conclusion in their study on the prevention of hepatocarcinogenesis promotion by FB<sub>1</sub>-glucose reaction products in rats.

The lesions observed on gross and microscopic examination of FB-exposed pigs were expected and agreed with several dietary studies in the literature (10–12, 42). The pale yellowish surface areas as well as the mild necrosis and disorganization of hepatic cords observed in the livers of two of the FB-G pigs could be explained by the concentration of unreacted FB in their diet (122  $\mu\text{mol/kg}$ ). Similar microscopic lesions have been observed in pigs fed 24  $\mu\text{mol}$  of FB<sub>1</sub>/kg of diet for 5 days (11)



or 42  $\mu\text{mol}$  of  $\text{FB}_1/\text{kg}$  of diet for 28 days (10), both lower than the FB concentration in the diet of our FB-G pigs.

In conclusion, the results of feeding dietary FB–glucose reaction products at 528  $\mu\text{mol}/\text{kg}$  of diet with 23% unreacted FB (122  $\mu\text{mol}$  of  $\text{FB}/\text{kg}$ ) suggest that these products could substantially protect swine in instances of widespread FB grain contamination. Additional research is required to explore the feasibility of this detoxification strategy in swine feed operations affected by FB contamination. Its implementation in the field will largely depend on the use of appropriate feed-processing equipment.

#### ABBREVIATIONS USED

AST, aspartate aminotransferase; BIL, total bilirubin; C-20, *d,l*-erythro-C20-dihydrosphingosine; CHOL, total cholesterol; DEN, diethylnitrosamine; ELEM, equine leukoencephalomalacia;  $\text{FB}_1$ , fumonisin B<sub>1</sub>;  $\text{FB}_2$ , fumonisin B<sub>2</sub>; FB, fumonisins ( $\text{FB}_1 + \text{FB}_2$ ); FB-G, fumonisin B–glucose; GGT,  $\gamma$ -glutamyl-transferase; NEB, nonenzymatic browning reaction; OPA, *o*-phthalaldehyde; PPE, porcine pulmonary edema; Sa, sphinganine; So, sphingosine; Sa/So ratio, ratio of sphinganine to sphingosine.

#### SAFETY

*Fusarium moniliforme* M1325 corn culture material contains fumonisin B<sub>1</sub>, a class 2B carcinogen. In consequence, it was handled accordingly.

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