Total Fumonisins Are Reduced in Tortillas Using the Traditional Nixtamalization Method of Mayan Communities[1,2]

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ABSTRACT Fumonisin B_1 (FB_1) is a maize mycotoxin. In tortilla preparation, maize is treated with lime (nixtamalization), producing hydrolyzed FB_1 (HFB_1) due to loss of the tricarballylic acid side chains. This study determined the following: *1*) whether nixtamalization by Mayan communities reduces total fumonisins, and *2*) the steps in the process at which reduction occurs. Tortillas prepared by the traditional process contained FB_1, FB_2 and FB_3 and their hydrolyzed counterparts. There were equimolar amounts of FB_1 and HFB_1 in the tortillas, but the total fumonisins were reduced 50%. The total FB_1 plus HFB_1 in the residual lime water and water washes of the nixtamal accounted for 50% of the total FB_1 in the uncooked maize. HFB_1 and FB_1 were present in a 1:1 mol/L ratio in the water washes of the nixtamal, the masa dough and the cooked tortillas, whereas the ratio of HFB_1:FB_1 in lime water after steeping was 21. Water washes contained 11% of the FB_1 that was in the uncooked maize. The results show that the traditional method reduced the total fumonisins in tortillas and reduced the sphinganine elevation (a biomarker closely correlated with fumonisin toxicity) in cells treated with extracts of tortillas compared with cells treated with extracts of contaminated maize. J. Nutr. 133: 3200–3203, 2003.

KEY WORDS: • fumonisin B_1 • hydrolyzed fumonisin B_1 • maize • nixtamalization • tortillas

Fumonisin B_1 (FB_1)[4] is a fungal contaminant of maize worldwide and is responsible for diseases of farm animals (1). It is a liver and kidney carcinogen in rodents and was recently evaluated to be possibly carcinogenic for humans (2). Fumonisins are not DNA reactive, and renal or liver damage is a prerequisite for carcinogenicity (3). Fumonisins (including FB_1, FB_2, and FB_3) are inhibitors of ceramide synthase, a key enzyme in the de novo sphingolipid biosynthesis pathway (1). Their toxicity and carcinogenicity are closely correlated with disruption of sphingolipid metabolism (3). Because toxicity is a prerequisite for carcinogenicity, a no observable effect level (NOEL) has been determined for both toxicity and carcinogenicity. A provisional maximum tolerable daily intake (PMTDI) of 2 μg/(kg body · d) for fumonisins B_1, B_2 and B_3, alone or in combination has been proposed (3). The PMTDI was calculated on the basis of a safety factor of 100 and a NOEL of 0.2 mg/(kg body · d) for the critical target (rat kidney). Processing methods that reduce the amount of fumonisins in food will reduce fumonisin intake, thereby protecting consumers from the possible adverse effects associated with exceeding the PMTDI.

In the preparation of tortillas, maize is treated with lime, which when heated hydrolyzes the tricarballylic acid side chains, reducing a portion of the FB_1 to the aminopentol backbone (HFB_1) (1). Alkali processing to prepare tortillas is practiced throughout the Americas including Guatemala, other parts of Central America, and Mexico. The process is known as nixtamalization (4). A similar process is used commercially in the United States (5), which has a growing population of immigrants from Mexico and Central and South America. Thus, understanding the comparative safety of nixtamalized products using traditional and commercial processes is of importance to consumers in the United States and elsewhere.

Nixtamalized maize products provide the majority of the daily energy for a large proportion of the population in the Central Highlands of Guatemala. A recent survey conducted in the Central Highlands (unpublished data) found that 100% of the population surveyed consumed maize as tortillas and that the mean daily consumption was 14 tortillas. The consumption of maize derived from products other than tortillas was ~145 g. Bressani (4) reported that the daily per capita maize consumption in Guatemala was 318 g. Because of the large amount of maize products consumed by communities in the Central Highlands of Guatemala, even relatively low levels of fumonisins in maize could pose a health risk. For example, at 1 μg total fumonisins/g of maize product, a 60-kg person consuming 318 g would exceed the PMTDI by a factor of 2.65 Several studies have shown that nixtamalization,

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[4] Abbreviations used: FB, fumonisin B; HFB, hydrolyzed fumonisin B; NOEL, no observable effect level; OPA, *ortho*-phthaldialdehyde; PMTDI, provisional maximum tolerable daily intake.

when conducted in laboratory or full-scale commercial settings, reduced the total fumonisin content of maize (6,7).

The purpose of this study was as follows: 1) to determine whether the traditional method of nixtamalization as practiced by rural Kaqchikel-speaking Mayan communities in the Central Highlands of Guatemala reduced the level of fumonisins in tortillas produced from fumonisin-contaminated maize, and 2) to determine the steps in the traditional process at which reduction in fumonisin levels were most likely to occur.

MATERIALS AND METHODS

Maize for preparing tortillas. Maize contaminated with fumonisins was obtained from North Carolina in 1999. The maize was shelled and segregated so that it contained predominately intact kernels and shipped to the INCAP where it was used to prepare tortillas using the traditional process of the rural Kaqchikel-speaking Mayan communities.

Preparation of tortillas. Maize was mixed and divided into three equal lots. Approximately 400 g from each lot was placed in steel pots, and samples were removed and stored frozen for analysis by HPLC for FB_1 and HFB_1 and by LC-MS for FB_2, FB_3 and HFB_2 and HFB_3. Coarsely ground lime (CaO, 82 g) was added to 1 L of water; 0.2 L of this stock lime solution was added to each pot followed by an additional 1.1 L of water. The lime water/maize preparation was boiled for ~1.75 h; as water evaporated, fresh water was added. The mixture was allowed to cool and steep for 15 h. The steep water was collected and the alkali-treated cooked maize (nixtamal) was rinsed three times with 1.1 L of water. The nixtamal was ground into masa. The volume of the steep water and the rinse water and the wet weight of the nixtamal and masa dough were recorded for each lot. Samples were removed and stored frozen for HPLC analysis. Tortillas were shaped by hand. The mean weight of an uncooked and cooked tortilla was 42 and 16 g, respectively. The tortillas were cooked on a comal (a dish made with clay) over a wood fire. Cooking temperatures varied from 170°C on the outer edge to 212°C at the center of the plate (4). Tortillas were typically 0.5–1 cm thick and ~10 cm in diameter. The time of cooking tortillas was ~3.5 min. A total of 10 tortillas were prepared from each lot. After drying at 38°C, the tortillas were weighed and stored frozen for later HPLC analysis of FB_1 and HFB_1 and for LC-MS analysis for FB_2, FB_3 and HFB_2 and HFB_3.

Analysis for FB_1 and HFB_1. After drying, 2–3 g of each tortilla (10/lot) and samples of the uncooked maize (1/lot) were extracted with 25 mL of acetonitrile/water (1:1; pH adjusted to 4.5) as described previously (7). For uncooked maize and cooked tortillas, a second acetonitrile/water extraction was done and samples from both extractions were derivatized using *ortho*-phthalaldehyde (OPA) (Pierce, Rockford, IL) according to previously described methods (8,9) and analyzed by HPLC with fluorescence detection (8,9). Alkaline steep water and water rinses were centrifuged (500 × *g* for 10 min) to remove solids and then acetonitrile/water 1:1 (600 μL) and OPA-derivatizing reagent (500 μL) were added directly to 50 μL of the clear liquid sample (pH 4.5) and analyzed by HPLC for FB_1 and HFB_1. LC-MS analysis was similar to that described previously (7) and was used to confirm the presence of other fumonisins. The analytical standard of FB_1 was prepared using the method of Meredith et al. (9), and the purity (>96%) was determined by the procedure of Plattner and Branham (10). The HFB_1 was prepared by the method of Poling and Plattner (11), and MS data were used to verify the purity of FB_1 and HFB_1 standards (9). Standards for HFB_2 and HFB_3 were provided by Ronald Plattner (USDA, Peoria, IL). Results of the HPLC analysis for FB_1 and HFB_1 are presented in nmol/g so that the amount of HFB_1 recovered in each fraction could be compared with the amount of FB_1 in the contaminated maize used to prepare the tortillas.

Bioassay method. To determine the ability of fumonisins in tortillas and maize to disrupt sphingolipid metabolism (a biomarker for fumonisin exposure and toxicity), a porcine renal epithelial cell line ($LLC\text{-}PK_1$) was used (12,13). The test agents were acetonitrile/water extracts of the fumonisin-contaminated maize used to prepare the tortillas and extracts of the tortillas prepared from the fumonisin-contaminated maize. Clean maize (<0.4 μg FB_1/g) and commercial masa flour (<1 μg FB_1/g) were used for comparison purposes. Because maize extracts from samples even of the highest quality are toxic to cells in culture, preliminary experiments were conducted to determine the dilution and dosing duration that would avoid the acutely toxic effects of concentrated maize extracts on cultured cells. Residues from 1:1 acetonitrile/water (pH 4.5) extracts (25 mL) of 1.5 g of clean or contaminated maize dissolved in 30 mL of growth medium had no effect on ATP-dependent dome formation or tight junction integrity after 6 h of exposure based on visual observation using phase contrast light microscopy. Therefore, 5-g samples of each test agent were extracted and the dried residue was dissolved in complete growth medium (12,13) and diluted to 30% in complete growth medium. Cells were exposed for 6 h and then harvested, extracted as described previously (13) and analyzed for free sphingoid bases (12), a biomarker for ceramide synthase inhibition and fumonisin toxicity (1).

Statistical analysis. Statistical analysis was done using Sigma Stat software (Jandel Scientific, San Rafael, CA). One-way ANOVA was used followed by tests for post-hoc multiple comparisons using Duncan's multiple range test. All data were expressed as mean ± SD, and differences among means were considered significant if $P \leq 0.05$.

RESULTS

The FB_1 concentration in the uncooked maize after two successive acetonitrile/water (1:1) extractions was 38.1 ± 7.4 μg FB_1/g (52.8 ± 7.4 nmol/g) of uncooked maize ($n = 6$). The FB_1 found in the second extraction of uncooked maize comprised 27 ± 7% of the total ($n = 6$). In tortillas, FB_1 and HFB_1 found in the second extraction comprised 24 ± 7 and 23 ± 9%, respectively ($n = 29$). The relative amounts of FB_1 and HFB_1 on a molar basis in the cooked tortillas were approximately equal (Fig. 1A). This was also the case in the uncooked masa dough in which the ratio of FB_1 to HFB_1, was 1.04 ± 0.39 ($n = 3$). On the basis of the LC-MS analysis, the ratio of the areas under the peaks for FB_1:FB_2:FB_3 (1:0.4:0.3) in the uncooked maize and the ratio under the peaks of FB_1, FB_2, FB_3 (Fig. 1B) and HFB_1, HFB_2 and HFB_3 (Fig. 1C) in the cooked tortillas did not differ.

The total fumonisins (nmol FB_1 plus nmol HFB_1) in the cooked tortillas accounted for approximately half (46.2 ± 7% $n = 3$) of the FB_1 that was in the uncooked maize. The total FB_1 in the lime water and water washes accounted for the other half (47.6% ± 11%, $n = 3$) of the total FB_1 originally present in the uncooked maize, and the three fractions (tortillas, lime water and washes) together accounted for 94 ± 18% ($n = 3$) of the FB_1 that was in the uncooked maize. A total of 11% of the FB_1 in the uncooked maize was accounted for in the combined water washes (Fig. 2A), however, the first two washes accounted for >90% of the total removed by rinsing (Fig. 2A). Although HFB_1 and FB_1 were present in approximately equimolar amounts in the uncooked masa dough, cooked tortillas and water washes of the cooked maize, the amount of HFB_1 in lime water after steeping was much greater than that of FB_1 (Fig. 2B).

Both the extracts of the FB_1 contaminated maize and the tortillas prepared from the contaminated maize caused significant elevation in sphinganine in $LLC\text{-}PK_1$ cells compared with the extracts of clean maize and commercial masa flour (Fig. 3). The elevation in sphinganine and the sphinganine to sphingosine ratio (Fig. 3 *inset*) caused by extracts of the cooked tortillas was reduced ~60% relative to that caused by the contaminated maize.

DISCUSSION

The nixtamalization process, regardless of the scale, appears to similarly reduce total fumonisins in the final product. Using

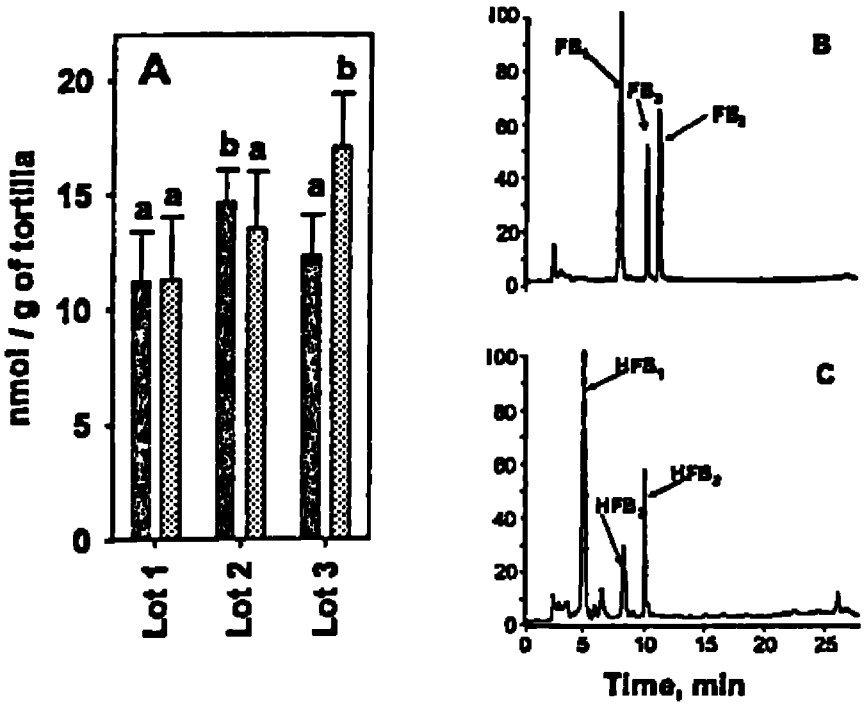

FIGURE 1 The total (*A*) and relative (*B*, *C*) amounts of fumonisin B_1 (FB_1; black bars) and hydrolyzed fumonisin B_1 (HFB_1; gray bars) in cooked tortillas expressed as nmol FB_1 and normalized to the calculated grams of uncooked maize required to prepare 1 g of tortilla. Values are means ± SD of 10 tortillas prepared from each lot of maize. FB_1 or HFB_1 means without a common letter are different, $P \leq 0.05$. (*B*, *C*) Examples of the relative amounts of fumonisins in acetonitrile/water (1:1) extracts of cooked tortillas based on single ion monitoring LC-MS analysis. The *y*-axis values are the percentages of the total ion current normalized to the highest peak.

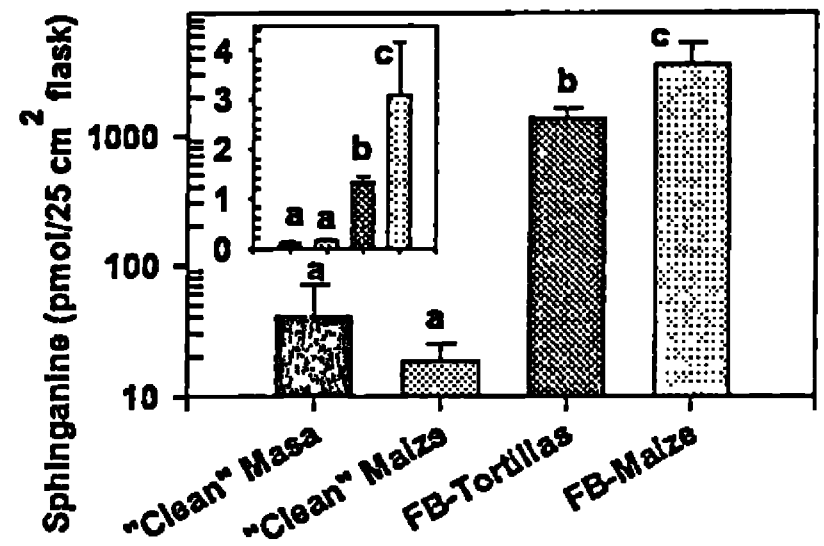

FIGURE 3 The ability of acetonitrile/water (1:1) extracts of commercial masa flour (Clean Masa), clean maize, fumonisin B_1–containing tortillas (FB-Tortillas) and the fumonisin B_1–containing maize (FB-Maize) used to prepare the tortillas to disrupt sphingolipid metabolism based on the elevation in sphinganine in LLC-PK_1 cells. The total FB_1 plus hydrolyzed fumonisin B_1 (HFB_1) concentration in the final growth medium was <1.3, <1.3, 7.3 and 15.8 μmol/L, respectively. The inset is the sphinganine to sphingosine ratio. Means without a common letter differ, $P \leq 0.05$, $n = 4$/treatment group).

the traditional nixtamalization and tortilla preparation process of the Mayan communities, the total FB_1 and HFB_1 content in cooked tortillas was reduced ~50% compared with the uncooked maize. This is very similar to the reduction seen using a full-scale commercial production line for preparing nixtamalized maize products (5) and although somewhat less than the reduction (81.5%) reported using a "pilot scale" nixtamalization procedure (6), the resulting reduction in total fumonisins is consistent in all three processing methods. The present study focused on FB_1; however, because the ratios of FB_1:FB_2:FB_3 were similar in the uncooked maize and the cooked tortillas, it can be concluded that all of the fumonisins of the B series are similarly decreased in the cooked tortillas.

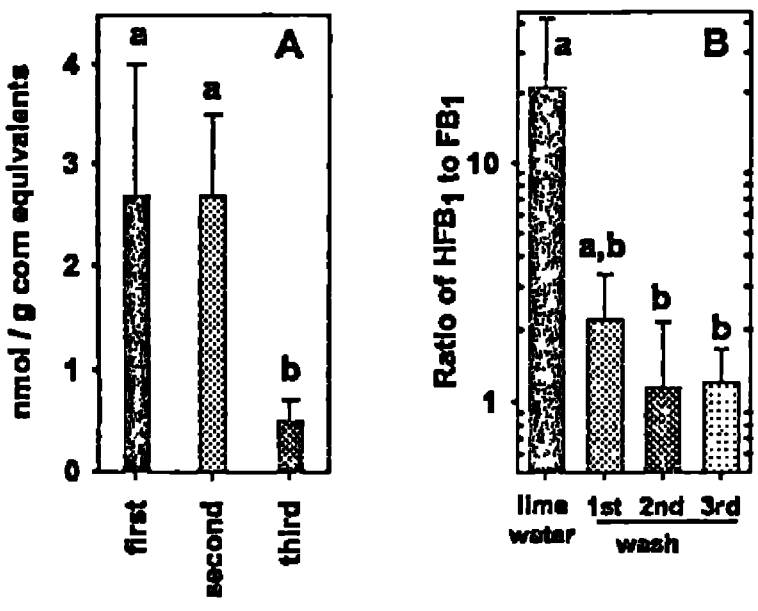

FIGURE 2 The total amount (*A*) and molar ratio (*B*) of fumonisin B_1 (FB_1) and hydrolyzed fumonisin B_1 (HFB_1) in each wash of the nixtamalized maize (*A*) and also in the lime water (B). Values are means ± SD in three successive washes (first, second, third) of the nixtamal. The values are normalized to the calculated grams of uncooked maize required to prepare the measured grams of nixtamal. Means without a common letter differ, $P \leq 0.05$, $n = 3$. For the comparison of "lime water" to the "first wash," $P = 0.0534$.

Regardless of the scale of the process, the initial cooking and steeping of maize under alkaline conditions is the most important step for converting FB_1 to HFB_1 and in reducing the total fumonisins in the cooked tortillas. In the commercial process, the cooking/steeping liquid was found to contain predominantly HFB_1 (7). Based on the data in Voss et al. (7), the ratio of HFB_1 to FB_1 in the cooking/steeping liquid was 45.5; in the pilot-scale study (6), the ratio was ≥20.2. These results are very similar to what was found with the process used by the Mayan communities in which there was ~21 times more HFB_1 than FB_1 in the lime water after cooking and steeping overnight (Fig. 2*B*).

In the study of Meredith et al. (8), the tortillas from two Guatemalan Central Highland communities (Santa Maria de Jesus and Patzicia) contained high levels (>10 μg/g) of total fumonisins. The actual maize used to prepare the tortillas was not available for analysis; however, based on the results of the present study, the levels of FB_1 in the unprocessed maize would have had to be >20 μg (total fumonisins)/g maize to have ≥10 μg/g in the cooked tortillas. Levels of FB_1 of 20 μg/g in maize are uncommon in commercial maize (3); thus the fact that 66% of the tortillas from Santa Maria de Jesus contained ≥10 μg/g total fumonisins indicates that the quality of the maize used to prepare tortillas is at times exceedingly poor.

There are two results from the 1999 report (8) that cannot be explained easily in light of the findings of the present study. First, the tortillas from the 1999 study (8) contained predominantly HFB_1, whereas in the present study, the levels of HFB_1 and FB_1 (on a molar basis) in tortillas are approximately equivalent (Fig. 1). Second, the nixtamal in the earlier study (8) contained predominantly FB_1, whereas in the present study, the ratio of HFB_1 to FB_1 (on a molar basis) in the masa (1.04 ± 0.39) was similar to the ratio in the tortillas after cooking. It was suggested by Meredith et al. (8) that perhaps the conversion of FB_1 to HFB_1 occurred during cooking of the tortillas, but this contention is not supported by the results of the present study or other studies (6,7) where it is the boiling of the maize in lime water that appears to be responsible for the conversion of FB_1 to HFB_1. The only plausible explanations for the findings in the earlier study of Meredith et al. (8) are that the tortilla samples were mishandled before analysis (i.e., microbial decomposition during shipment or storage) or

that the process of nixtamalization as practiced in Mayan communities can be highly variable, leading to highly variable results. Of the 50 household samples collected in 1995 (8), only frozen samples were analyzed. Thus, microbial activity during shipping or storage contributing to the high levels of HFB_1 is unlikely. In 1995, Santa Maria de Jesus experienced a water shortage that resulted in water rationing (8). If the amount of water used for alkaline treatment (boiling and steeping) had been reduced and washing steps omitted, then much higher levels of HFB_1 might be expected in the cooked tortillas.

Regardless of the reason for the high levels of HFB_1 in tortillas in the earlier study, the results of the present study show clearly that the process of nixtamalization as practiced by Mayan communities under optimal conditions (adequate water) can effectively reduce the level of total fumonisins in cooked tortillas to a degree similar to that seen in studies using commercial or pilot-scale processes. In addition, the biological activity of the acetonitrile/water extract of the cooked tortillas was reduced relative to extracts of the uncooked maize based on the elevation in sphinganine, which is closely correlated with fumonisin toxicity in vitro (13) and in vivo (1). Furthermore, boiled maize foods such as porridge (Africa) or polenta (Italy) have been shown to reduce FB_1 by 23 and 8%, respectively (14), whereas boiling plus alkali treatment in this study reduced the total fumonisins by 50%, and ~50% of the total fumonisins in the tortillas was present as hydrolyzed fumonisins. In other studies, even greater reductions in total fumonisins were reported (6,7). Given greater reduction in total fumonisins through alkali treatment and the fact that hydrolyzed fumonisins are less toxic in both rats (15) and mice (16) than the parent compounds, we hypothesize that at a given level of FB_1 contamination of maize, nixtamalized tortillas will be (on a gram for gram basis) a safer product than boiled maize products prepared from the same contaminated maize. This finding is important because fumonisins, including hydrolyzed fumonisins, have been suggested to be potential risk factors for neural tube defects in humans in areas in which consumption of maize products, including tortillas, is high.[5] Therefore, minimizing fumonisin exposure through appropriate processing at the household level in these areas is an important management strategy for reducing the number of consumers who potentially exceed the recommended PMTDI.

[5] Workshop on the Role of Fumonisins in Neural Tube Defects, January 2003, Atlanta, Georgia (Marasas, W.F.O., Riley, R. T., Hendricks, K. A., Stevens, V. L., Sadler, T. W., Gelineau-van Waes, J., Missmer, S. A, Cabrera Valverde, J., Torres, O. L., Gelderblom, W.C.A., Allegood, J., Martínez de Figueroa, A. C., Maddox, J., Miller, J. D., Starr, L., Sullards, C., Roman Trigo, A. V., Voss, K. A., Wang, E. & Merrill, A. H., Jr. Fumonisins disrupt sphingolipid metabolism, folate transport, and development of neural crest cells in embryo culture and *in vivo*: A potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize).

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