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MULTIMYCOTOXIN ANALYSIS IN URINES TO ASSESS INFANT EXPOSURE: A CASE STUDY IN CAMEROON

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ABSTRACT

This study was conducted to investigate mycotoxin exposure in children (n=220, aged 1.5-4.5 years) from high mycotoxin contamination regions of Cameroon and to examine the association between the mycotoxin levels (in total 18 analytes) and several socio-demographic factors and anthropometric characteristics. A cross-sectional study was conducted in six villages in Cameroon with 220 children. Mycotoxins and their metabolites were detected in 160/220 (73%) urine samples.

There were significant differences in the mean contamination levels of ochratoxin A ($p = 0.01$) and β-zearalenol ($p = 0.017$) between the two agro-ecological zones investigated. Likewise significant differences were observed in the mean levels of aflatoxin M_1 ($p = 0.001$) across the weaning categories of these children. The mean concentration of aflatoxin M_1 detected in the urine of the partially breastfed children (1.43 ng/mL) was significantly higher ($p = 0.001$) than those of the fully weaned children (0.282 ng/mL).

Meanwhile, the mean concentrations of deoxynivalenol (3.0 ng/mL) and fumonisin B_1 (0.59 ng/mL) detected in the urine of the male children was significantly (p value 0.021 for deoxynivalenol and 0.004 for fumonisin B_1) different from the levels detected in the urine of female children; 0.71 ng/mL and 0.01 ng/mL for deoxynivalenol and fumonisin B_1 respectively. In this study, there was no association between the different malnutrition categories (stunted, wasting and underweight) and the mycotoxin concentrations detected in the urine of these children.

However, there is sufficient evidence to suggest that children in Cameroon under the age 5 are exposed to high levels of carcinogenic substances such as fumonisin B_1, aflatoxin M_1 and ochratoxin A through breastfeeding. To the best of our knowledge, this is the first report of its kind carried out in West Africa to determine multi-mycotoxin exposure in infants.

Key words: mycotoxins, biomarkers, children, urine, weaned, breastfeeding

1.1 Introduction

The environment of children during early development (from in utero to 36 months) is critical for disease and risk in later life. Children are uniquely vulnerable to environmental toxicants because of their greater relative exposure, less developed metabolism, and higher rates of cell division, growth, and development (Makri et al., 2004). The environmental insults of childhood may manifest themselves over a lifetime of growth to adulthood and senescence. In addition to physiologic vulnerabilities, children may have great social vulnerabilities as well as poverty and malnutrition. In most third world countries especially in Sub-Saharan Africa (SSA), many children are not only malnourished, but they are also chronically exposed to high levels of toxic fungal metabolites (mycotoxins) in their diet (Cardwell et al., 2004). The fact that developmental exposure to agents such as mycotoxins produces effects that differ qualitatively and quantitatively from those produced by adult exposure, represents a major empirical and conceptual foundation for child health risk assessment (NAS, 1993; Carlson, 1998).

Traditionally, mycotoxin exposure in humans has been assessed by detecting the presence of the mycotoxins in food commodities (Song et al. (in press)). This approach is generally considered less accurate since the mycotoxins are not homogeneously distributed within any given food commodity (Bennett and Klich, 2003; Scott and Trucksess, 1997; Tanaka et al., 2010). Secondly, there are some other routes through which individuals can be exposed to mycotoxins such as physical contact through the skin and inhalation. Furthermore, in case of disease outbreak for which mycotoxin exposure was suspected, the food is most often no longer available for analysis. As a result, the traditional evaluation method is only suitable to assess the exposure of populations to some toxins or to identify possible risk groups. It cannot really reflect accurate information on the individual intake of mycotoxins (Kuiper-Goodman, 1999).

In order to circumvent the above mentioned shortcomings, biomarkers have been proposed as a suitable alternative. Recent developments in molecular biology and instrumentation have provided new tools for use in environmental health care research and biological risk assessment. Biomarkers have the potential to be quantitative dosimeters of individual exposure and biologic effective dose, as well as early warning signals of biologic effect (Montesano et al., 1997; Ramjee et al., 1992; Riley and Pestka, 2005; Turner et al., 2000; WHO, 2001; Wild and Gong, 2010).

Prior to this study, these same authors investigated mycotoxin contamination in three agricultural staples (maize, peanuts and cassava) from three agro-ecological zones of Cameroon over two seasons (July-August 2009 (season 1) and December-January 2010-2011 (season 2)). Data form this survey revealed a high mycotoxin contamination rate in two of the three agro-ecological regions. A summary of the contamination data is presented in Table 1.

The aim of this study was to investigate the relationship between some anthropometric data as well as dietary habits and mycotoxin exposure in these children in order to better understand the causes of exposure and to be able to set-up effective prevention campaigns. Up till now, most mycotoxin exposure assessments in developing countries have focused on the aflatoxins (AFs) and fumonisin B_1 (FB_1). Moreover, for aflatoxin exposure, aflatoxin-albumin adduct is usually the target metabolite in blood. Obtaining blood samples from younger children (< 5 years) creates a high barrier for participation. It was for this reason that urine was chosen as target matrix. It was therefore our objective to push beyond the frontiers and include some other analytes such as citrinin (CIT), zearalenone (ZEN), ochratoxin (OTA), deoxynivalenol (DON), T2-toxin (T2) and their metabolites which are frequent contaminants of food and which have so far not been investigated. The choice of the different mycotoxin metabolites (potential biomarkers) for the different analytes was based on toxicokinetics data extracted from the literature as recently described by Njumbe Ediage et al. (2012).

1.2 Materials and methods

1.21 Reagents and materials

LC-MS grade methanol, high performance liquid chromatography (HPLC) grade acetonitrile, methanol and n-hexane were purchased from VWR International (Zaventem, Belgium). Bond Elut strong anion exchange (SAX) SPE cartridges were obtained from Varian (Sint-Katelijne Waver, Belgium). Water was purified on a Milli-Q Plus apparatus (Millipore, Brussels, Belgium).

1.2.2 Standards

Mycotoxin-reference standards and metabolites namely aflatoxin B_1 (AFB_1), HT-2 toxin (HT-2), OTA, ZEN, FB_1, deepoxy-deoxynivalenol (DOM), CIT, alpha zearalenol (α-ZOL) and beta zearalenol (β-ZOL), were purchased from Sigma-Aldrich (Bornem, Belgium). T-2 toxin was purchased from Biopure (Tulln, Austria). DON and aflatoxin M_1 (AFM_1) were purchased from Fermentek (Jerusalem, Israel). Ochratoxin alpha (OTα) was obtained from Coring System Diagnostix (Gernsheim, Germany). 4 hydroxy OTA (4-OH OTA) was a gift from Professor Ralf Blank (Institute of Animal Nutrition and Physiology, Kiel, Germany). Stock solutions of DON, AFM_1, AFB_1, HT-2 toxin, T-2 toxin, OTA, ZEN, FB_1, CIT, α-ZOL and β-ZOL were prepared in methanol at a concentration of 1 mg/mL. DOM and OTα were obtained as solutions, 100 μg/mL and 10.3 μg/mL respectively in acetonitrile. DON-3Glu was synthesized using the protocol described by Wu et al. (2007) while AFB1-N7-guanine adduct (AFB_1-N7Gua) was prepared using the procedure described in Egner et al. (2006). Hydrolyzed FB_1 (HFB_1 or aminopentol) was synthesized by alkaline hydrolysis as described in Pagliuca et al. (2005). ZEN-Glu was obtained as a gift from Dr. Alfons Callebaut (Veterinary and Agrochemical Research Center, Tervuren, Belgium)

1.2.3 Participants recruitment

Two agro-ecological zones were targeted and this included the humid forest with monomodal rainfall (HFM) and the western highland (WH) of Cameroon. The selection of these two zones was based on an earlier study which revealed a high occurrence of mycotoxins in three different dietary staples (maize, peanuts and cassava) originating from these two zones. An overview of the contamination data for the different (three) agro-ecological zones is presented in Table 1. From each of the target (two) agro-ecological zones, a region was selected. These agro-ecological zones can also be referred to as the Northwest and Southwest regions representing the HFM and WH regions respectively. Three villages within each of these two regions were chosen on the grounds there exists a health center in the village or that the health center was within the nearest 15 km. This was to reduce the time between sample collection and storage since most of the selected villages were situated in very remote areas of the country. The villages include Anta (ANTA), Baga (BB) and Bmanya (BM) representing the Northwest region while Diffa (DIFFA), BGwana (BGN) and Kake (KII) represented the Southwest region. A schematic view of the map of Cameroon, showing the geographical location of the studied villages is represented in framework in Figure 1.

Ethical approval and clearances for this study were obtained from the Ministry of Public Health in Cameroon to cover the two regions. Another ethical approval was obtained from Ghent University. Forty children per village (in total 240 children), aged 1.5 to 4.5 years, were envisaged for recruitment with the inclusion of one child per household. Children on medication or having medical record of kidney or metabolic problems were excluded from the study. The parents or guardians of the selected children were informed about the nature of the study. During the information session, the objectives were clearly read out in the presence of the mother and the child and also in the presence of some local interpreters in cases where the

mother was not well educated to understand the language of the informant. A day or two was allowed for the parents or guardian to decide on their participation in the project. Once they agreed to participate, they were asked to sign an informed consent form. The fieldwork for the study (sample and questionnaire collection) took place in March 2012.

1.2.4 Questionnaire

A questionnaire was administered by trained interviewers (nurses of the health centers). The information collected from the children included age, gender, weaning status, type of long term or short term illnesses. Concerning the parent or guardian, information on the age and weight of the mother, socioeconomic status such as the level of education of the mother, occupation of the mother, average monthly income of the mother and specific food choices were recorded. A lot of concern was placed on the parent or guardian status because it was seen as more relevant to the nutrition and wellbeing of the child. The educational status of the mother was classified into three categories: no school education, primary school education or secondary school education. The food consumption details of the entire family were also recorded. The parent or guardian was asked to indicate which of the three staples (maize, peanut and cassava) was most frequently used to prepare the family meal, if the staples were locally produced or were bought from the local markets, the type of processing carried out, the frequency of consumption by the family on a weekly basis of the selected staples, and the number of times per day that the same staples was eaten by the child.

A 24h dietary recall was also performed where by all the main and minor ingredients used in preparing the home meal were clearly listed so as to identify the possible route(s) of contamination and/or exposure of the child(ren) concerned.

1.2.5 Age, weight and height measurements

The ages of both the parents and/or guardian and children were extracted from official birth certificates. The weight and height of all the children were measured using accurately calibrated instruments (electronic scales: Soehnle, max wt 40 kg, accurate 10g; height measurement SCHORR, Maryland, USA). From the recorded heights and weights, three types of calculations were performed namely the weight for age Z-score (WAZ), the height for age Z-score (HAZ) and weight for height score (WHZ) according to the recommendation of the World Health Organisation Multicentre Growth Reference Study Group (WHO, 2006). According to this WHO criteria, a Z-value of < -2 for any of these criteria (WAZ and HAZ) is recognized as malnutrition and < -3 as severe malnutrition. Meanwhile a WHZ score reflects the body shape, where a low value is indicative of wasting and acute malnutrition . The height and weight measurements were carried out by nurses who were all qualified health personnel.

1.2.6 Urine sample collection and multi-mycotoxin analysis

First morning void urine was demanded from all volunteers. A 60 mL urine recipient was distributed to each of the participating child a day prior to the day of sample collection. Urine samples were frozen within six hours after collection. In some cases, urine samples were rejected if it took more than 6hr from the time of collection to the time of storage (freezing at -20^{o}C). Stability studies carried out prior to this survey by this same group of researchers revealed that the target analytes were stable for 12hr at 25°C post-collection (Njumbe Ediage et al., 2012). All samples were later preserved in dry ice (-56°C) and transported to the Laboratory of Food Analysis, Ghent University, Belgium by airway.

In total 220 samples met the strict specifications of collection and storage and were analyzed. A 10 mL urine was used for mycotoxin analysis. All samples were analyzed by using a

validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method previously described by Njumbe Ediage et al. (2012) using liquid-liquid extraction (LLE) in combination with SAX SPE clean-up.

1.2.7 Statistical Analysis

Where the urinary analyte was not quantifiable, middle value between zero and the analyte limit of quantification was used. The data were not normally distributed, hence equal variance was not assumed. Non parametric tests such as Mann-Whitney U and Kruskal Wallis tests were performed to investigate possible associations or differences between the mycotoxins concentrations and the age groups, gender, weaning status and agro-ecological zones. All the analyses were performed using SPSS Software version 20.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p<0.05$.

1.3 Results

1.3.1 Descriptive data on the characteristics and staple foods of the children

Demographic data for the 220 children are presented by village in Table 2. With respect to gender, BGwana had the least number of males (n=4) which was significantly different from the other villages (average n=24). The fact that BGwana had the smallest population recruit (n=14) compared to the other villages (average n=41) can explain the fewer number of male recruit. However, there were no statistical differences in the mean age distribution across the six villages ($p > 0.05$).

With respect to the weaning status, the children were classified in three categories as follows: wholly breastfed, partially breastfed or fully weaned (i.e. no breast milk). The distribution (percentage) of the children within the different breastfeeding categories for entire sample population was as follows: wholly breastfed 15%, partially breastfed 25%, while 60% were fully weaned. Meanwhile Table 2 also shows the percentage of totally weaned and wholly breastfed children for the six different villages. In most villages, children $\geq$ 2 years were either partially breastfed or fully weaned. Each parent/guardian was asked through the questionnaire to indicate which of the three staples (maize, peanuts and cassava) was mostly used to prepare the family meal. Only one of the three staples could be selected from the list. Maize was used as the main ingredient by 34% of the participants on a weekly basis to prepare the family. For cassava, 50% indicated that this staple was frequently used on a weekly basis while only 16% of the households indicated the use of peanuts as the main ingredient. Table 2 further illustrates per village, the number of households who indicated the use of one of these staples on a weekly basis to the total number of households recruited from that particular village.

Significant differences in the consumption pattern for maize, cassava and peanuts were observed across the two agro-ecological zones. In the Northwest region representing the western highland, 67/126 (53%) of the participants indicated that maize was used as the main staple on a weekly basis, while for the Southwest region only 8/94 (9%) of the households used maize as the main staple. With respect to cassava consumption, 44/126 (34%) of the households from the Northwest region indicated cassava as the main ingredient in the family meal as against 70% (66/94) for households from the Southwest region (Table 2).

In general peanuts were never considered as the main ingredient for the family meal. Only 15/126 (12%) households from the Northwest region indicated the use of peanuts to prepare the family meal as against 21% for the households of the Southwest region (Table 2).

Table 3 shows the prevalence of malnutrition in the children population recruited for this study. There was a strong prevalence of stunted growth (low HAZ), low weight for age Z-score (WAZ) and wasting in 39%, 37% and 23% respectively of the children according to the World Health Organization 2006 criteria (WHO, 2006). The mean values for each of the three growth parameters WAZ, HAZ and WHZ were below zero (mean = -1.63, -1.1, -1.36, respectively) with particularly low values for WAZ and WHZ. An average of 17% of the children had a Z-score of < -3 in at least one parameter, a situation regarded as extreme malnutrition by WHO criteria.

A great majority (85%) of the parents/guardian had only primary school education and this was reflected in their average monthly income which was less than 50 United States dollar (USD).

1.3.2 Distribution of mycotoxin contamination and co-occurrence rate in the urine samples

Mycotoxins were detected in 160/220 (73%) of the samples analyzed. The analytes investigated and their corresponding limit of detection (LOD) were as follows; AFB_1 (0.83 ng/mL), CIT (2.88 ng/mL), T-2 toxin (T-2, 0.05 ng/mL), HT-2 toxin (HT-2; 0.42 ng/mL), HFB_1 (0.51 ng/mL), DOM (0.65 ng/mL), aflatoxin AFB_1-N7Gua (0.85 ng/mL), OTA (0.03 ng/mL), OTα (0.03 ng/mL), 4-OH OTA (0.12 ng/mL), DON (0.04 ng/mL), AFM_1 (0.01 ng/mL), FB_1 (0.01 ng/mL), ZEN (0.1 ng/mL), β-ZOL (0.01 ng/mL), α-ZOL (0.31 ng/mL), DON-3Glu (2.25 ng/mL) and ZEN-Glu (3.65 ng/mL).

Seven of the 18 analytes investigated were detected in one or more samples. The analytes included OTA, DON, AFM_1, FB_1, ZEN, β-ZOL and α-ZOL. The occurrence frequency for the detected analytes were as follows: OTA (32%), DON (17%), AFM_1 (14%), FB_1 (11%), β-

ZOL (8%), ZEN (4%), α-ZOL (4%) and DON-3Glu (1%). Data regarding the individual mycotoxin concentration were further investigated in terms of co-occurrence in the samples analyzed. The co-occurrence rate of 2, 3 and 4 co-occurring mycotoxins per sample was 35%, 5% and 5% respectively.

1.3.3 Correlation between the different urinary concentrations and socio-demographic factors and anthropometric characteristics

A Mann-Whitney test revealed significant differences in OTA ($p = 0.01$) and β-ZOL ($p = 0.017$) mean concentrations between the two agro-ecological regions. For both mycotoxins, the mean concentrations detected in children from the Northwest region (0.25 ng/mL and 1.82 ng/mL for OTA and β-ZOL respectively) were significantly higher than those from the Southwest region (0.16 ng/mL and 0.31 ng/mL for OTA and β-ZOL respectively). For the other mycotoxins, no significant differences in the mean concentrations were observed between the two regions. A pairwise comparison of the mycotoxin concentration for the individual villages was further computed. Again, a Kruskal-Wallis test revealed significant differences in OTA ($p = 0.008$) and FB_1 ($p = 0.003$) concentrations across the different villages. The boxplots of Figure 1 show the distribution of each of these toxins for the different villages, while Table 4 highlights which of the villages were significantly different from the others as indicated by the p values. All villages were compared pairwise.

A more critical insight analysis of the data was performed in order to exploit the possible correlation/associations between the different analyte concentrations and some socio-demographic factors. Table 5 shows the geometric mean concentration, the minimum and maximum concentration of each analyte for the different demographic categories. Meanwhile,

the mean concentrations of DON and FB_1; 3.0 ng/mL and 0.59 ng/mL respectively detected in the urine of the male children was significantly (p value 0.021 for DON and 0.004 for FB_1) different from the levels detected in the urine female children; 0.71 ng/mL and 0.01 ng/mL for DON and FB_1 respectively.

The mean concentration of the different mycotoxins was statistically the same across the different age group categories ($p > 0.05$). To perform this comparison, the children were classified into four groups based on their ages: group 1 comprised of children between 1 to < 2 years, group 2 was made up of children between 2 to < 3 years, children between 3 to < 4 years were placed in group 3, while group 4 was made of children older than 4 years but less than 5 years old.

A Kruskal Wallis test was performed to investigate possible differences between the respective mean mycotoxin concentrations and the different malnutrition and weaning categories. No differences were observed between the mean concentrations of the analytes and being underweight (low WAZ), wasted (low WHZ) or stunted (low HAZ) ($p > 0.05$). However, significant differences were observed between the weaning categories of the children and mean AFM_1 concentrations detected in the urine. The mean AFM_1 concentration detected from the fully weaned children (0.282 ng/mL) was significant different from those who were partially breastfed (mean concentration 1.43 ng/mL, $p = 0.035$) and wholly breastfed (mean 0.69 ng/mL, $p = 0.001$). There were no significant differences in the mean AFM_1 concentration between the wholly breastfed and the partially weaned children ($p = 0.226$). Exposure was highest in the partially breastfed group while the fully weaned infants had the least AFM_1 contamination.

However, there were no significant differences in the mean OTA, DON, FB_1, ZEN, α-ZOL and β-ZOL concentrations detected in the urine samples across the different weaning categories.

1.4 Discussion

The order of occurrence (%) of the analytes OTA (32%), AFM_1 (14%), FB_1 (11%) in samples from Cameroon was the same to those reported in a similar study carried out with Sri Lanka adult population (Desalegn et al. 2011). This convergence of results could be due to similar occurrence pattern of the parent toxins in foodstuffs native to these countries; taking into consideration that both countries are situated within the same latitude (0-10°N).

The differences in the urinary concentrations of DON and FB_1 between the male and female children could be due to a random occurrence or the fact that males in general eat more than females and hence they are more exposed. However, this necessitates further studies before any definite conclusion(s) can be made. Any conclusion drawn at this phase of the research might be misleading.

Since there were differences in food preferences between the two agro-ecological zones (highlighted in Table 2), it was anticipated that this could result in differences in the mycotoxin contamination pattern in the urine of children from these two agro-ecological zones. While FB_1, AFB_1, DON, and ZEN (in that order) are said to be the most frequent contaminants in cereals from SSA (e.g. maize) (Njobeh et al., 2010), AFB_1 is a common contaminant in peanuts and cassava products. OTA is more of a contaminant in peanut products (Njumbe Ediage et a1., 2011) than in products derived from the other two food matrices (maize and cassava flour). Despite the high frequency of consumption of peanuts for households from the Southwest region (20/94) than those from the Northwest region (15/126) (Table 2) and the 70% occurrence rate of OTA in urine of children from two villages (Kake and BGwana) in the Southwest region, the mean concentration of OTA detected in urine of children from the Northwest region was significantly higher than those from the Southwest

region ($p = 0.01$). This observation raises questions regarding other dietary sources which contributed significantly to the urinary levels of OTA other than peanuts. Hence there is need for further investigation to identify the other possible sources of OTA exposure across the studied communities. However, the occurrence rate of OTA in the urine samples from the other villages was less than 20%.

Investigations carried out on human biological fluids have shown that OTA contamination is widespread in several European and developing countries. OTA was reported in human milk in Norway, Sweden, Germany, Italy, Chile, Australia and Brazil (Scott, 2005; Munoz et al., 2010). Since the mean concentration of OTA detected in the urine of wholly breastfed children (0.19 ng/mL) was essentially the same with those of the fully weaned children (0.16 ng/mL) and partially breastfed children (0.21 ng/mL); there is no doubt that the wholly breastfed infants tend to have the same exposure risk as the fully weaned children. These findings support the possibility of dietary recommendations to women during lactation aimed at tentatively reducing infant exposure to this carcinogen substance.

Furthermore, none of the target metabolites of OTA such as OT alpha and 4-OH OTA were detected in the urine samples. Similar findings were also reported for the Korean population (Ahna et al., 2010). However, as suggested by Munoz et al. (2009, 2012) interindividual variability in the detoxification of OTA in human urine may account for the observed variations in urinary OT alpha and the possibility cannot be excluded that a low rate of OTA detoxification is a characteristic of some populations.

Comparing our results with other studies from different populations, in terms of contamination levels; the mean concentration detected in this study; 0.2 ng/mL are higher than the mean concentration detected in Hungary; 0.013 ng/mL (Fazekas et al., 2005), Italy; 0.02 ng/mL (Pena et al., 2006), Bulgaria; 0.05 ng/mL and 0.168 ng/mL (Petkova-Bocharova et al., 2003) but lower than those detected in Croatia; 2.3 ng/mL (Domijan et al., 2003). The

highest concentration of OTA reported so far in human urine was detected in Sierra Leone with a range of 0.07–148 ng/mL (no mean was reported) (Jonsyn, 2000). In general biomonitoring studies carried out in Europe often report a high prevalence rate (>60%) of OTA in human urine than in studies carried out in SSA. This could be due to differences in dietary habits.

Furthermore, dietary aflatoxin exposure of West African children has been linked primarily to consumption of contaminated maize and peanuts (Njobeh et al., 2010; Allen et al., 1992). AFB_1 was not detected in any of the 220 urine samples analyzed which is contradictory with results earlier reported in West Africa (Gong et al., 2002; Jonsyn, 2000) where high levels of aflatoxin-albumin adduct was frequently detected in the serum of children. This discrepancy in results is due to the relatively long half-life of the AFB_1-albumin adduct. The half-life of human albumin is ~20 days. AFB_1-albumin may theoretically accumulate following chronic exposure to reach levels 30-fold higher than that the levels found after a single dose. In contrast, urinary AFB_1 excretion will tend to parallel intake over the previous few days (Scholl et al., 2006).

The sampling period (March, 2012) coincided with the start of the planting season, a period during which peanuts and maize are not readily available at the household level, which can possibly explain the zero (0%) occurrence rate. Furthermore, the high limit of quantification for AFB_1 (2 ng/mL) with the present analytical method made the possibility for trace detection very difficult. In a recent study by Tchana et al. (2010), conducted with malnourished children from Cameroon, the maximum AFB_1 detected in urine of these children was 0.15 ng/mL. Going by these results, it can be said that the detection limit for AFB_1 necessitate further improvement (using a more sensitive instrument) to render it more suitable to biomonitor AFB_1 in human urine especially in populations which are not chronically exposed to AFB_1.

AFM_1, a hydroxylated form of AFB_1 showed a 14 % occurrence rate with a significant difference in the mean concentration of AFM_1 between the wholly breastfed and the fully weaned ($p = 0.001$). The partially breastfed were more exposed (mean concentration 1.43 ng/mL) compared to the fully weaned children (0.282 ng/mL). From this cross-sectional study, it could be deduced that weaning status rather than age could be a strong determinant of AFM_1 exposure in children of less than 5 years. Based on the facts represented herein, we hereby assume that the sources of AFM_1 exposure could have been either from breast milk for the wholly breastfed children and/or complementary milk products for the partially weaned infants. Interestingly, in earlier studies reported by Polychronaki et al. (2008) and Gong et al. (2003) with Guinean and Gambian children, significantly higher urinary aflatoxin levels were detected with the fully weaned children compared to the partially breastfed children. This result differs from our present findings. The reasons for this discrepancy in results is presently unclear. An earlier report by Polychronaki et al. (2007) raised the possibility that urinary AFM1 may not only reflect ingested AFM1 originating from breast milk. On the the hand, very sensitive detection systems such as fluorescence detection and enzyme-linked immunosorbent assay (ELISA) as used in the studies of Polychronaki et al. (2008) and Gong et al. (2003) allowed detection at very low picogram/mL levels and hence possibly led to an increase in the power of their statistical prediction. It is worth noting that despite being a weaker carcinogen, AFM1 is as cytotoxic as AFB1 (Neal et al., 1998).

Compared to other studies conducted in SSA; the levels of AFM1 detected in samples from Cameroon (0.06-4.7 ng/mL) were 80-fold lower than levels reported in samples from Gambia (0.1-374 ng/mL) (Wild et al., 1991). Studies conducted in Europe have always shown low urinary levels of AFM1. This is true since the parent compound (AFB1) is not a major contaminant in European foodstuffs as compared to dietary staples from SSA.

As zearalenone and its metabolites, mainly α-ZOL and β-ZOL, are potent estrogenic compounds and can adversely affect reproduction in laboratory and farm animals at higher doses, they are considered as potential endocrine disruptors (Metzler et al., 2010). ZEN, α-ZOL and β-ZOL were detected in 8/220 (3.6%), 9/220 (4%) and 18/220 (8%) of the samples, respectively.

There was no co-occurrence of α-ZOL and β-ZOL in any of the samples. This clearly illustrates inter-individual variability in the metabolism of ZEN. However, co-occurrence of ZEN and β-ZOL was detected in one sample. In this sample the ratio of ZEN to β-ZOL was 1:4. The high frequency of occurrence of β-ZOL compared to α-ZOL in the urine samples confirms a similar observation that was made in one of our previous studies (Njumbe Ediage et al., 2012). The presence of α-ZOL in 4% of the samples is worth noting since the estrogenic activity of α-ZOL is greater than that of ZEN (3-4 times) and β-ZOL (10 times). Interestingly, the occurrence rate of ZEN and its metabolites (α-ZOL and β-ZOL) was highest in children from the Northwest region where there was a high frequency of maize consumption. To the best of our knowledge, this is the first study carried out to determine exposure of SSA children to ZEN and its metabolites.

Comparing these results with the study carried out in New Jersey (USA) (Bandera et al., 2002), the mean concentration of total ZEN (including its metabolite) was <0.21 ng/mL which was lower than the levels reported in this study (3.47 ng/mL). This calls for the need to further investigate the effect of ZEN exposure on the target population especially with respect to reproductive health.

DON is a frequent contaminant of maize and maize products. Exposure of SSA children to DON has so far not yet been evaluated. A recent review of epidemiological data highlighted the possibility of DON-induced gastroenteritis, growth faltering and immunotoxicity in humans with as consequence a possible increase on the susceptibility to infectious diseases

(Pestka and Smolinski, 2005). So far, the human health consequences of DON exposure remain largely unexamined. Though this study failed to establish a possible association between DON exposure and the specific food preferences for the different agro-ecological regions, a 17% occurrence rate and a maximum urinary concentration of 77 ng/mL should raise some concern.

The frequency of contamination of total DON reported from other European countries is often the double of what was obtained in this study; 31% for Spain (Rubert et al., 2011) and 33% for Italy (Solfrizzo et al., 2011). Exceptionally high occurrence rate of 69% and 99% was reported in Portugal (Cunha et al., 2011) and the United Kingdom (Turner et al., 2011) respectively. In the latter, the highest concentration of DON detected in the urine samples was 78.2 ng/mL which is similar to the maximum concentration detected in this study (77 ng/mL).

In order to determine if the tolerable daily intake (TDI) was exceeded by the child with 77 ng/mL detectable urinary DON, the model proposed by Meky et al. (2003) was used. This model assumes the following (1) the concentration of DON in the morning urine samples was representative of the whole day (2) the average volume of urine excreted per day was 0.2 L (for a 15.2 kg child) (3) 35 % of the ingested DON was being excreted in urine and (4) all DON originated from the previous 24 h intake only. Hence the sample with the above mentioned concentration would translates to a TDI of 2.89 µg/kg body weight/day which exceeds the provisional maximum TDI of 1 µg/kg body weight/day as proposed by Scientific Committee on Food (SCF, 2002). This calls for appropriate measures to address this situation as children under the age of 5 constitute the most vulnerable group.

Since most of the children with detectable levels of DON were either partially breastfed or fully weaned, it is difficult to conclude if significant levels of DON are transferred into breast milk. One of the detoxification metabolite of DON (DON-3-Glu) was detected in 1% (2/220) of the samples analyzed.

Just like DON, FB_1, is also a most frequent contaminant of maize and maize products. Based on the results of our previous survey (Table 1) we anticipated a high occurrence rate of FB_1 in the weaning foods (which normally consist of maize porridge) and hence a high incidence or occurrence of this analyte in the urine of these infants. However, only 11% of the samples analyzed were found positive for this analyte. The mean levels of FB_1 detected were not significantly different between the different weaning categories. FB_1 was detected in the urine of four (1.8%) children fed exclusively on breast milk with levels between (0.73-1.3 ng/mL). This suggest the fact that FB_1 could be carried-over in human breast milk though at low concentrations. Studies carried out to evaluate FB_1 carry-over to milk are conflicting. A few authors have reported low but detectable levels of FB_1 in bovine milk (Maragos et al., 1994; Spotti et al., 2001). The lack of sufficient data on FB_1 carry-over in human milk could be due to the lack of sensitive analytical methods for this matrix (Gazzotti et al., 2009). In this study, the analytical method used had as detection limit < 1ng/mL for FB_1 which could be considered sensitive enough to detect very low concentrations of this toxin in urine. The levels of FB_1 detected in the urine were low (mean 2.98 ng/mL) with one sample detected with a concentration of 48 ng/mL. Using the same model as described for DON, this translates to a TDI of 0.71 μg/kg bw/day which is however less than the 2 μg/kg bw/day as proposed by Scientific Committee on Food (SCF, 2000b).

Data from other international studies reveal an almost negligible presence of this analyte in different human urine samples. In Portugal none of the 68 samples had detectable levels of FB_1 nor FB_2 (Liliana et al., 2011). Similar results were obtained from Austria (Warth et al., 2011), Korea (Ahn et al., 2010) and Spain (Rubert et el., 2011). Data from Mexico revealed a 74% occurrence rate with a mean urinary concentration of 0.035 ng/mL (Gong et al., 2008).

In general very limited number of studies has been carried out to assess urinary levels of FB_1. This can be attributed to the fact that not until recently (from 2008 onward) biomonitoring of

the sphinganine/sphingosine ratio was considered as a suitable biomarker to assess exposure to fumonisins rather than monitoring of the parent analyte (FB_1).

1.5 Strengths and limitations

The major strengths of this study was the diversity in the age and gender distribution across the study group, differences in the food preferences for the different agro-ecological zones, the diversity in the weaning status and the differences in socio-economic status to assess possible correlations between mycotoxin concentrations. This is further strengthened by the view that almost all the different classes of mycotoxins were included (represented) in this study.

A potential weakness of this study could be the relatively small sample size (n=220) which may have compromised the statistical prediction power of the study and may have likely influenced the associated statistical inference (confidence interval, standard errors, standard deviation, p-value). The inability to detect aflatoxins in the low pictogram/mL levels may have compromised the prediction power of the study design. It is therefore imperative to further validate this study design (for example using a very sensitive instrumentation) and re-evaluate the associated statistical inferences with a much larger sample population. Likewise the inability to detect the glucuronides of DON (<2%) and ZEN might necessitate further investigation in order to provide possible explanations.

1.6 Conclusion and recommendation

Young children (< 5 years of age) raised in high mycotoxin contaminated regions (zones) of Cameroon are exposed to significant levels of multiple toxins during infancy. There was

sufficient evidence to suggest a possible transfer of FB_1, AFM_1 and OTA via breast milk to these children. However, since potential adverse health effects of mycotoxins in children in Cameroon are not well established, this study could be considered as a pilot study to raise awareness of the potential health consequences these young children may suffer from during a later stage of their lives.

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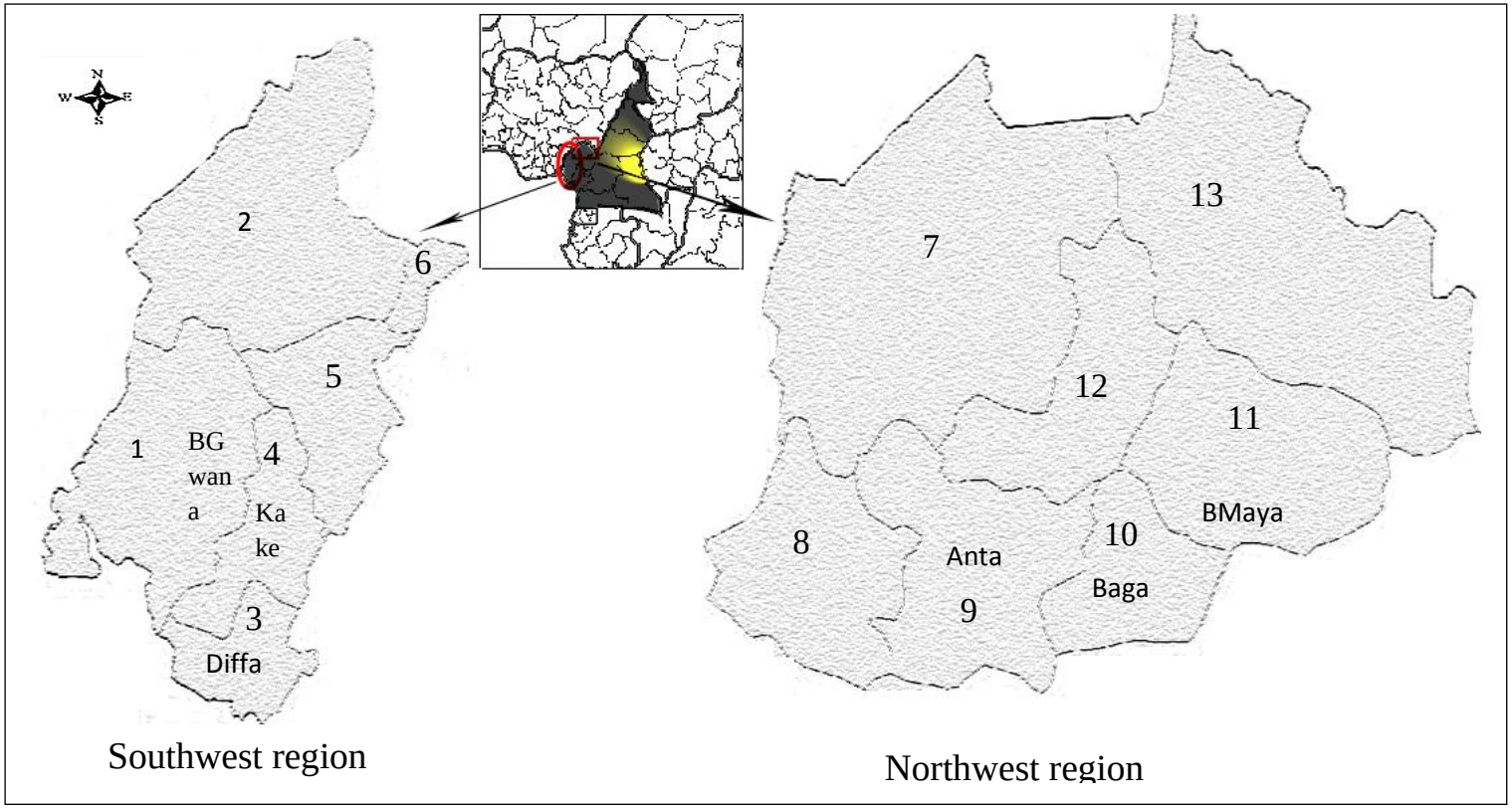
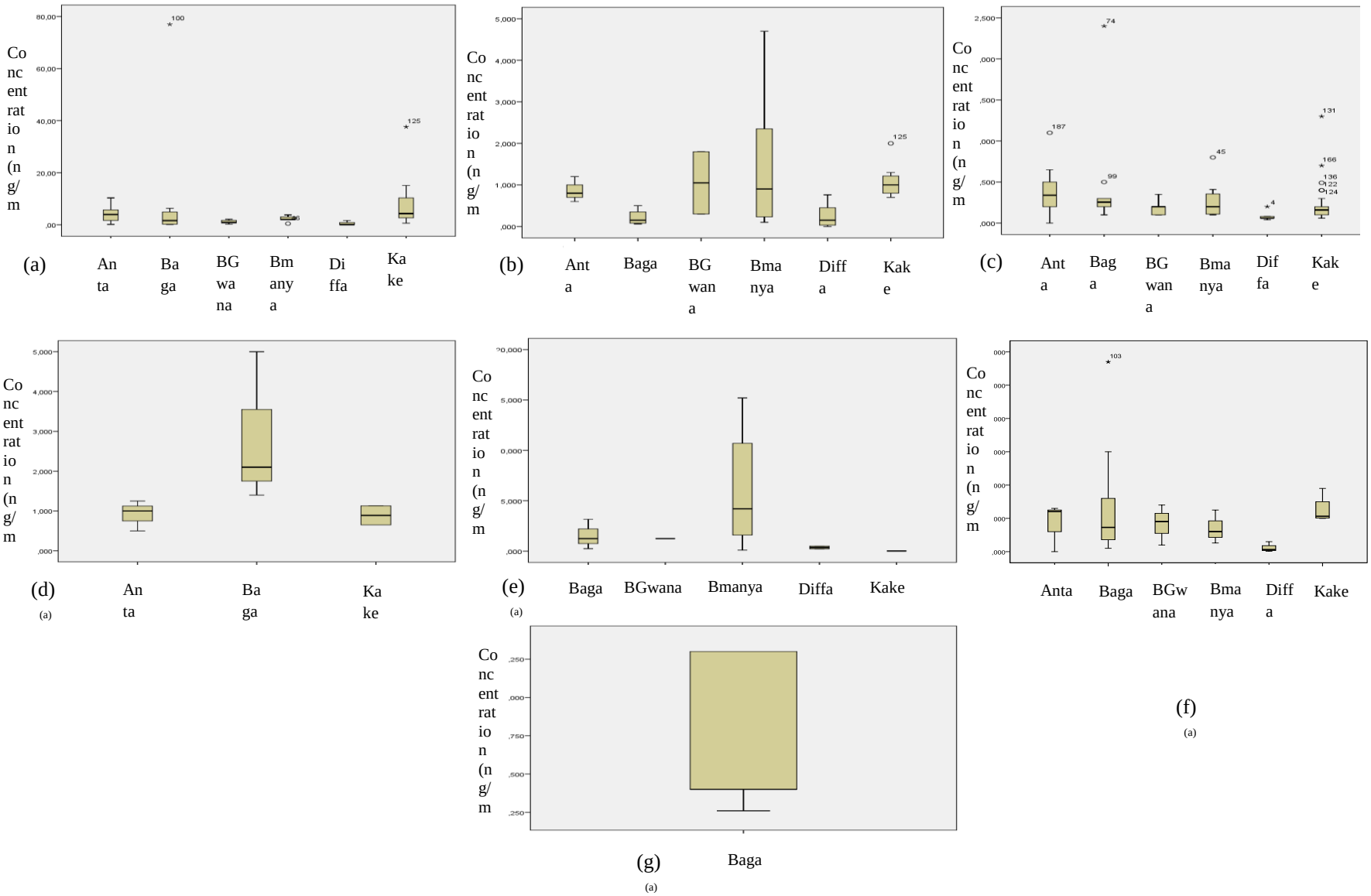
Figure 1



The numbers represent the different divisions that make up the different regions. 1; Ndian, 2; Manyu, 3; Fako, 4; Meme, 5; Kupe Manenguba, 6; Lebialem, 7; Menchum, 8; Momo, 9; Mezam, 10; Ngo-Ketunjia, 11; Bui, 12; Boyo and 13; Donga Mantung.

Figure caption:

Map of Cameroon showing the location of the different studied regions (Northwest and Southwest) and villages.


Concentration (ng/m
(a)
Anta
Baga
BGwana
Bmanya
Diffa
Kake
(b)
Anta
Baga
BGwana
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Diffa
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(c)
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(d)
(a)
Anta
Baga
Kake
(e)
(a)
Baga
BGwana
Bmanya
Diffa
Kake
Anta
Baga
BGwana
Bmanya
Diffa
Kake
(f)
(a)
(g)
(a)
Baga

Figure 2

Figure caption

Figure 1: Boxplots showing the distribution of seven different mycotoxins for the six different villages investigated: (a) Deoxynivalenol, (b) Aflatoxin M_1, (c) Ochratoxin A (d) Zearalenone, (e) Beta zearalenol, (f) Fumonisin B_1 without outlier (sample contaminated with 48 ng/mL), (g) Alpha zearalenol.

Table 1. Summarized data on the occurrence of mycotoxins in different dietary staples from Cameroon

	Mean concentration (CI, 95%) (µg/kg) of mycotoxins detected in the different staples							
	[a]Season 1				[b]Season 2			
Sample matrix	Agroecological zones							
Maize	HFM	HFB	WH	Mean total	HFM	HFB	WH	Mean total
Deoxynivalenol	1134 (<LOQ-2141)	234 (212-918)	1567(<LOQ-2741)	978	1876(<LOQ-2411)	459 (<LOQ-1084)	2341 (218-3842)	1559
Fumonisin B_1	1467 (75-3716)	543 (<LOQ-1418)	2178 (112-5412)	1396	1845 (<LOQ-4030)	435 (314-2841)	2654 (<LOQ-3212)	1645
Fumonisin B_2	1187 (112-2268)	419 (<LOQ-843)	987 (75-2882)	864	1895 (10-2890)	467 (<LOQ-915)	1567 (112-1846)	1309
Fumonisin B_3	398 (<LOQ-1442)	167 (75-482)	355 (65-412)	300	755 (112-2180)	489 (132-864)	336 (<LOQ-698)	543
Zearalenone	168 (75-279)	191 (<LOQ-228)	189 (85-262)	182	165 (35-334)	174 (<LOQ-242)	270 (55-286)	203
3 and 15 acetyl deoxynivalenol	66 (65-231)	46 (<LOQ-187)	90 (54-170)	67	78 (<LOQ-115)	84 (<LOQ-176)	98 (<LOQ-186)	86
Roquefortine C	78 (<LOQ-94)	66 (<LOQ-137)	98 (<LOQ-145)	81	81 (<LOQ-84)	89 (<LOQ-118)	103 (<LOQ-181)	81
Beauvericine	162 (<LOQ-412)	151 (<LOQ-	131 (<LOQ-264)	148	111 (<LOQ-312)	141 (<LOQ-	129 (<LOQ-	127

		384)				284)	385)	
Peanuts								
Ochratoxin A	ns	ns	ns	ns	6 (<LOQ-12)	3 (<LOQ-10)	4 (<LOQ-4)	4
Aflatoxin B_1	ns	ns	ns	ns	78 (<LOQ-125)	58 (<LOQ-77)	61 (<LOQ-110)	66
Cassava								
Aflatoxin B_1	87 (<LOQ-194)	36 (<LOQ-95)	78 (6-193)	67	95 (<LOQ-125)	25 (<LOQ-32)	89 (<LOQ-141)	70
Penicillic acid	71 (<LOQ-184)	27 (<LOQ-96)	35 (<LOQ-72)	44	29 (<LOQ-76)	28 (<LOQ-46)	27 (<LOQ-44)	42

CI: confidence interval. HFM: humid forest region with monomodal rainfall, HFB: humid forest region with bimodal rainfall, WH: western highland. ns: not sampled
LOQ: limit of quantification. The LOQ for deoxynivalenol, 3+15 acetyl deoxynivalenol, fumonisin B_1, fumonisin B_2, fumonisin B_3, zearalenone, roquefortine C, ochratoxin A, Penicillic acid and aflatoxin B_1 were 27 µg/kg, 54 µg/kg, 0.3 µg/kg, 1 µg/kg, 100 µg/kg, 27 µg/kg, 1 µg/kg, 0.3 µg/kg, 25 µg/kg, 6 µg/kg for peanuts and 1 µg/kg for cassava respectively.
[a]Season 1: July-August 2009
[b]Season 2: December-January 2010-2011

Ta

	Villages					
	Villages belonging to the Northwest region			Villages belonging to the Southwest province.		
Characteristic	Anta	Baga	Bmaya	Diffa	BGwana	Kake
Number of children per village	N=40	N=43	N=43	N=35	N=14	N=45

Age (months)[a]	37.6 (17-54)	31.3 (18-54)	37.3 (15-54)	40.1 (17-54)	41.6 (24-53)	36.1 (16-54)
Sex (male:female)	23:17	25:18	24:19	20:15	4:10	28:17
% of fully weaned infants (%)	42	74	63	60	55	68
% of wholly breastfed infants (%)	2.27	2.73	1.82	3.18	2.27	2.73
Maize consumption[b]	39:40	6:43	22:43	5:35	3:14	0:45
Cassava consumption[b]	1:40	22:43	21:43	30:35	11:14	25:45
Peanut consumption[b]	0:40	15:43	0:43	0:35	0:14	20:45

[a]Mean (95% confidence interval (CI)). [b]Maize, cassava and peanut consumption refers to the number of households who indicated the use of one of these staples on a weekly basis to the total number of households who participated for the study per village.

Table 3 Prevalence of malnutrition in children based on the World Health Organization guidelines

Criteria[a]	Total	Prevalence rate (%)	
		Malnutrition[b]	Extreme malnutrition[c]
Wasting and acute malnutrition (WHZ)	220	23	15

Underweight (WAZ)	220	37	13
Stunting and chronic malnutrition (HAZ)	220	39	24

[a] Diagnosis of wasting based on weight for height Z-score (WHZ); underweight based on weight for age Z-score (WAZ); stunting based on height for age Z-score (HAZ) according to the WHO 2006 criteria.
[b] Malnutrition is defined as less than -2 for any of the three parameters.
[c] Extreme malnutrition is defined as less than -3 for any of the three parameters.

Table 4 Pairwise comparison of the different villages using the p-value(s)

Villages		p value	
		Mycotoxins	
		OTA	FB_1
Diffa			
	Bmanya	0.022*	0.05
	Baga	0.006*	0.903
	BGwana	0.021*	0.451
	Kake	0.015*	0.001*
	Anta	0.01*	0.641
Bmanya			
	Baga	0.679	0.110
	BGwana	0.551	0.704
	Kake	0.455	0.935
	Anta	0.308	0.534
Baga			
	BGwana	0.173	0.684
	Kake	0.061	0.008*
	Anta	0.435	0.234
BGwana			

	Kake	0.807	0.213
	Anta	0.047*	0.345
Kake			
	Anta	0.017*	0.456

*indicates pairs for which significant differences were observed (p value < 0.05). OTA: ochratoxin A, FB_1: fumonisin B_1

Table 5 Distribution of mycotoxin contamination by age, sex and agro-ecological zones

Variable/category	No	Mycotoxin contamination level: geometric mean (95% CI) (ng/mL) [a]						
		OTA	AFM_1	DON	FB_1	ZEN	β-ZOL	α-ZOL
Total (n=220)	220	0.2 (0.04-2.4)	0.33 (0.06-4.7)	2.22 (0.1-77)	2.96 (0.06-48)	0.97 (0.65-5.0)	1.52 (0.02-12.5)	0.98 (0.26-1.3)
Age group								
1-2 years old	43	0.18 (0.06-1.3)	0.46 (0.04-4.7)	1.58 (0.43-3.8)	0.36 (0.1-7.3)	1.16 (0.65-2.1)	nd	0.26
2-3 years old	54	0.18 (0.04-0.51)	0.22 (0.08-2.35)	3.94 (1.22-15.08)	2.49 (0.4-48)	1.12 (1.10-1.3)	1.31 (0.1-10.7)	nd
3-4 years old	68	0.25 (0.07-2.4)	0.71 (0.2-4.7)	2.3 (0.6-77)	0.53 (0.1-1.3)	2.38 (1.13-5)	0.64 (0.02-15.2)	0.72 (0.4-1.3)
4-5 years old	55	0.24 (0.1-0.5)	0.37 (0.1-1.13)	2.36 (0.15-5.5)	0.29 (0.06-1.3)	1.32 (1.25-1.4)	0.93 (0.02-6.5)	0.72 (0.4-1.3)
Gender								
male	126	0.19 (0.06-2.4)	0.57 (0.06-4.7)	3.0 (0.05-77)	0.59 (0.06-50)	0.86 (0.5-1.4)	0.76 (0.02-15.2)	0.59 (0.4-1.3)
female	94	0.16 (0.04-0.8)	0.35 (0.1-1.8)	0.71 (0.06-3.50)	0.02 (0.02-5.7)	1.13	2.1 (1.250-4.2)	0.58 (0.26-1.3)
Zone								
Northwest province	107	0.25 (0.1-2.4)	0.3 (0.06-4.7)	2.14 (0.1-77)	0.74 (0.1-7.3)	0.44 (0.5-5)	1.82 (0.1-15.2)	0.59 (0.26-1.3)
Southwest province	113	0.16 (0.06-1.3)	0.36 (0.06-4.7)	2.37 (0.43-15.08)	1.49 (0.3-48)	0.86 (0.65-1.13)	0.31 (0.02-1,25)	nd
Weaning status								
Wholly breastfed	33	0.19 (0.1-0.65)	0.69 (0.6-1.2)	2.98 (0.15-10.3)	1.03 (0.73-1.3)	0.85 (0.5-1.25)	0.86 (0.02-6.5)	nd
Partially breastfed	55	0.21 (0.06-0.49)	1.43 (0.7-4.7)	4.47 (1.22-37.6)	0.91 (0.26-1.9)	0.65	1.11 (0.02 – 15.2)	nd
Fully weaned	132	0.16 (0.08-2.4)	0.28 (0.08-1.8)	3.52 (0.21-77)	0.63(0.1-50)	1.25 (1.1-1.8)	1.13 (0.23-12.5)	0.59

[a]Mean (95% confidence interval (CI)). OTA: ochratoxin A, AFM_1: aflatoxin M_1, DON: deoxynivalenol, FB_1: fumonisin B_1, ZEN: zearalenone, β-ZOL: beta zearalenol and α-ZOL: alpha zearalenol. nd: not detected. Limit of detection for OTA, AFM_1, DON, FB_1, ZEN, α-ZOL and β-ZOL were 0.03 ng/mL, 0.01 ng/mL, 0.04 ng/mL, 0.01 ng/mL, 0.1 ng/mL, 0.31 ng/mL, 0.01 ng/mL respectively.