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Multiple mycotoxin exposure of infants and young children via breastfeeding and complementary/weaning foods consumption in Ecuadorian highlands

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ABSTRACT

The dietary exposure to mycotoxins in Ecuadorian children aged 0–23 months (320 rural and 603 urban) was evaluated based on the intake of breast milk and staple cereals used as complementary/weaning foods. A probabilistic distribution approach by first order Monte Carlo simulation was adopted to assess the locally occurring mycotoxins (aflatoxins M_1 and B_1 in breast milk, ochratoxin A and deoxynivalenol in wheat noodles and oat flakes, and HT-2 toxin in polished rice). Overall, exposure was modest but higher for rural children due to their monotonous diet. Aflatoxin exposure by breast milk intake were of health concern in both areas (Margin of Exposure and Combined Margin of Exposure Index < 10,000). Mycotoxin exposure by staple cereals intake was considered tolerable across feeding stages for individual mycotoxin-cereal combination (Hazard Quotient < 1) and combined exposure (Hazard Index < 1). The major exposure was to HT-2 toxin by rice intake at complementary feeding (15% rural and 4% urban above TDI) and at weaning stage (26% rural and 6% urban above TDI). Since the usual Ecuadorian diet is based on the same staple cereals, risk management actions could lead to a better protection of young children and also ensure higher safety of the recommended breastfeeding practices by protecting nursing mothers.

1. Introduction

Mycotoxins contaminate the diet of a large proportion of the world's population. Dietary exposure to mycotoxins might be higher in developing countries because of several conditions such as favorable environment for fungal growth and mycotoxin production; reliance on subsistence farming, and poor quality monitoring and enforcement of regulations in local markets (Shephard, 2008; Wild and Gong, 2010).

Infants and young children are particularly at risk and are about three times more susceptible than adults to the adverse effects of mycotoxins due to their higher intake/body weight ratio, higher metabolic rate and lower detoxification capacity (Hulin et al., 2014; Sherif et al., 2009).

The mycotoxin risk in children depends on the magnitude and frequency of exposure (Blankson and Mill-Robertson, 2016). A high risk is expected when consuming monotonous cereal-based diets that are typically contaminated with several mycotoxins (Cheli et al., 2014). At infancy, another potential dietary source of exposure is breast milk due to the possible lactational transfer of several mycotoxins and their metabolites from maternal diet (El-Tras et al., 2011). From those, the hydroxylated metabolite aflatoxin M_1 (AFM_1) is one of the major occurring mycotoxins in breast milk together with its carcinogenic precursor aflatoxin B_1 (AFB_1) (Gürbay et al., 2010; Turconi et al., 2004).

As in other Latin American (LA) countries, cereals are the most important complementary foods for infants and young children in Ecuador (Leonard et al., 2000). A rather low degree of contamination and co-occurrence of the major mycotoxins of health concern in the main staple cereals in Ecuador (polished rice, wheat noodles and oat

Abbreviations: AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; BMDL, lower confidence limit of bench mark dose; DON, deoxynivalenol; HI, Hazard Index; HQ, Hazard Quotient; LOD, limit of detection; MOE, margin of exposure; MOET, combined margin of exposure index; PMTDI, provisional maximum tolerable daily intake; PTDI, provisional tolerable daily intake; OTA, ochratoxin A

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flakes) was previously reported (Ortiz et al., 2013a). Despite this however, a risk of chronic exposure might be expected as well as a hazard from combined exposure to multiple mycotoxins (Assunção et al., 2015).

This study is the first report of dietary exposure to co-occurring mycotoxins in infants and young children aged 0–23 months in Ecuador and LA countries. The exposures to HT-2 toxin through polished rice; deoxynivalenol (DON) and ochratoxin A (OTA) through wheat noodles; DON and OTA through oat flakes, and AFM_1 and AFB_1 through breast milk intake were evaluated according to the child feeding pattern and as combined exposure. To prioritize risk management strategies, risk characterization was assessed comparing the estimated daily exposures to the reported toxicological levels for chronic exposure of individual mycotoxins. For combined exposure to multiple mycotoxins, the component-based approach based on concentration-addition method was applied.

2. Material & methods

2.1. Food consumption data

Data on the consumption of staple cereals, i.e. rice, wheat noodles and oat flakes, were collected from a total of 998 children aged 0–23 months, 348 from a rural canton (Nabon) and 650 from an urban canton (Cuenca), Azuay province, at the southern Ecuadorian highlands. These data were part of a cross-sectional survey conducted from June to September 2008 to evaluate nutritional status in the Ecuadorian highlands. This study was approved by the Ethics Committee of the University Hospital of Ghent, Belgium (Approval code B67020084011), and the Ethics Committee of the Central University of Quito, Ecuador (N° CBM/COBI 001-08). The sample size of the study was computed to detect a difference of 100 kcal d^{-1} in energy intake between the urban and rural setting, with a statistical power of 90%, type I error of 5% and assuming a 20% of non-response.

The selection of the participants was previously described (Ortiz et al., 2013b). Briefly, the rural canton Nabon is located in the country side at 3000 m above sea level. It has a considerable territorial dispersion which complicates the access the different communities (Municipal, 2012). Rural households were randomly selected from the census register of the children under 24 months from all communities of the canton. The urban canton Cuenca is the third largest city in Ecuador and it is the capital of the province. Cuenca is located at approximately 2550 m above sea level and at 70 km from Nabon (Guía-Oficial, 2012). There was no child register available in the urban canton and therefore, a cluster random sampling scheme was adopted using residential blocks as primary sampling unit. All households belonging to a selected block were visited door-to-door and the surveys were conducted without restriction in the number of infants that could be found per block.

In both settings, individual consumption data were obtained from the primary child caregivers at their homes using 24-h dietary recall questionnaires. Duplicate 24-h recalls were carried out in the urban area, while a single 24-h recall was conducted in the rural area due to limited access of the communities. To estimate portion sizes, each respondent was asked to fill a household recipient with the actual amount of food consumed by the child. This amount was determined in volume (mL) by trained interviewers and then converted into grams using recalled data of the consistency of each food consumed. Detailed recipe data were also collected to calculate the actual amount of consumed rice, wheat noodles and oat flakes in each of the composite dish. Breast milk intake was estimated assuming a proxy conversion factor of 13.5 g of milk per minute of breastfeeding (Da Cunha et al., 2013; Mills and Tyler, 1992). Data entry was done using Lucille food intake software (Ghent-University, 2010; Ochoa-Avilés et al., 2014) which allowed estimating of food intake at ingredient level, based on pre-set food composition databases.

The consumption of the cereal-based staples foods and breast milk per day was calculated and expressed as kg per kg^{-1} body weight (bw) using data from individual weight measurements. Children from who body weight data were not available were excluded from the analysis (n = 75). In total, the exposure assessment was carried out for 923 children, 603 children from the urban area and 320 children from the rural area. The dietary exposure assessment on daily bases was performed separately for children of the urban and rural area. In addition, children were grouped in three feeding pattern categories: group 1 (urban n = 61; rural n = 72): children being exclusively or predominantly breastfed (only water and water-based drinks, vitamins, minerals and medicines could be consumed besides breast milk) (PAHO/WHO, 2003); group 2 (urban n = 303; rural n = 163): children at complementary feeding stage (intake of cereals, cereal products and breast milk); and group 3 (urban n = 239; rural n = 85): children at weaning stage (no breast milk intake).

In the urban area, the individual usual dietary intake was determined from the duplicate 24-h recalls using the Multiple Source Method (MSM) program® (Harttig et al., 2011), that considers the intra-individual variability in consumption. The MSM outputs for habitual consumers were used to construct the distribution of consumption data. In the rural area, no MSM computation was applied and the distributions were constructed based on only one 24-h recall of the consumer population.

2.2. Food contamination data

The co-occurrence of ten mycotoxins of health concern in the main staple cereals identified from the food consumption surveys (polished rice, wheat noodles and oat flakes) was previously assessed and described (Ortiz et al., 2013a). Briefly, samples of polished rice (n = 125) were collected from May to July 2010 (rainy season) from the biggest rice mills in Ecuador located at the lowlands of the coastal region, which are the rice suppliers of the whole country. Samples of oat flakes (n = 70, 9 rural and 61 urban) and wheat noodles (n = 128, 15 rural and 113 urban) were collected during February–March 2010 from open markets and supermarkets of the same areas where food consumption surveys were conducted. About half of the urban samples were randomly selected for multimycotoxin analysis, whereas all rural samples were analyzed (polished rice n = 46; wheat noodles n = 80 and oat flakes n = 42). Co-occurrence of aflatoxin B_1, B_2, G_1 and G_2, OTA, DON, fumonisin B_1, zearalenone, and HT-2 and T-2 toxin was analyzed by UHPLC/TOFMS (Ortiz et al., 2013a). No contamination of aflatoxin B_2 and G_2, fumonisin B_1, zearalenone and T-2 toxin were found in none of the samples. In addition, OTA was analyzed in extra batches of oat flakes samples (n = 35) and wheat noodles (n = 59) by HPLC-FLD that was more sensitive for this mycotoxin (Ortiz et al., 2013a). All cereal samples were analyzed as dried raw material.

In the present study, the occurrence of AFB_1 and AFM_1 in breast milk was additionally evaluated. This analysis was carried out as part of a pilot study conducted from November 2012 to January 2013. Breast milk samples (n = 78) were obtained by self-expression of volunteer nursing mothers from the rural canton Nabon. Samples were collected in sterile plastic containers, transported at 4 °C and then frozen within 1 day at −20 °C until mycotoxin analysis. The analytical procedure is described as follows.

2.2.1. Analysis of AFB_1 and AFM_1: chemicals and reagents

LC grade water, acetonitrile, methanol, phosphate-buffered saline (PBS) solution, AFM_1 standard solution in acetonitrile (10 μg mL^{-1}) and standard of solid pure extract of AFB_1 were supplied by Sigma–Aldrich (St. Louis, MO, USA). Acetic acid (glacial) was supplied by Merck KGaA (Darmstadt, Germany). Easi-extract® Aflatoxin immunoaffinity columns were purchased from R-Biopharm Rhône (Glasgow, Scotland). The standard of AFB_1 was reconstituted using acetonitrile. Aliquots of standard stock solutions in acetonitrile (0.1 μg mL^{-1}) were dried under a gentle stream of nitrogen and stored

at −20 °C. Dried standards were reconstituted with a mixture of acetonitrile/water, 1:1 (v/v) and further dilutions were freshly prepared with the same solvent mixture.

2.2.2. Analysis of AFB_1 and AFM_1: sample treatment

Breast milk samples were skimmed with a mild-heat treatment at 35–37 °C for 10 min in a water bath (Memmert. Munich, Germany), followed by centrifugation for 15 min at 2403 g (Hettich EBA 20. Tuttlingen, Germany). Finally, the lower layer was filtered (Whatman N°4) (Galvano et al., 2008). A volume of 10 mL of skimmed sample was applied to immunoaffinity clean-up columns (IAC) (Easi-extract® Aflatoxin, R-Biopharm Rhône) at 2–3 mL min^{-1}, after bringing the IAC to room temperature and conditioned with 3 mL of PBS solution. The IAC was washed with 10 mL of PBS solution followed by 10 mL of water at a flow rate of 5 mL min^{-1}. AFM_1 and AFB_1 were eluted by gravity using 1.5 mL of the mixture methanol/acetonitrile, 2:3 (v/v) after 30 s of contact of the IAC with the solvent, and applying back flushing for three times. After drying the IAC with air stream, the eluate was finally diluted with 1 mL of water passed by gravity through the IAC. The sample was filtered (0.45 μm filter) and a volume of 20 μL was injected into the HPLC system.

2.2.3. Analysis of AFB_1 and AFM_1: instrumental parameters and analysis

AFM_1 and AFB_1 were analyzed on an Agilent 1200 HPLC system (Agilent Technologies, USA) consisting of an isocratic pump, vacuum degasser, autosampler, column oven (35 °C), and equipped with a Zorbax Eclipse C18 column (5 μm, 4.6 × 250 mm, Agilent Technologies, USA). An isocratic elution was applied with a mobile phase containing a mixture of acetic acid 2%/acetonitrile/methanol 40:35:25 (v/v/v) at a flow rate of 0.8 mL min^{-1}. Fluorescence detection was carried out at 365 and 450 nm of excitation and emission wavelengths, respectively. Quantification was performed by measurement of the peak areas at the retention time of AFM_1 (4.1 ± 0.04 min) and AFB_1 (5.3 ± 0.01 min) and comparing them with a six-point calibration curve (1–15 μg L^{-1}, $R^2 = 0.998$). The method performance parameters were evaluated following a single-laboratory optimization considering the minimum performance parameters for quantitative methods (Taverniers et al., 2004). Recovery experiments were performed in duplicate at 3 concentration levels (10, 15, 20 μg L^{-1}). The limits of detection (LOD) and quantification (LOQ) were calculated based on a signal-to-noise ratio 3:1 and 6:1, respectively. The intra-day precision was assessed based on the replicates of the recovery experiments, while inter-day precision was assessed by spiking a testing sample before extraction of at 10 μg L^{-1} of AFM_1 and AFB_1 during three consecutive days.

2.3. Dietary exposure assessment

A probabilistic distribution approach following a first order Monte Carlo simulation was adopted to assess the dietary exposure to mycotoxins. For food contamination data, the nature of the data did not allow distribution fitting as many non-detected values were found and limited values to attribute a good distribution fitting (Vinci et al., 2012). Therefore, non-detected values (< LOD) were replaced by half of the limit of detection (medium bound value) and the mean concentration was calculated for each mycotoxin for those values above LOD. In order to compare the average exposure between urban and rural children across different feeding stages, only the medium bound scenario was assumed for this study. Mycotoxins that occurred in only one of the samples were excluded from the exposure assessment. Data on daily consumption were fitted to probability distributions. The selection of the best fitting distribution was based on the lowest chi-square statistic and on inspection of probability–probability (P–P) plots. The dietary exposure distributions were modeled using first order Monte Carlo simulation based on 10,000 iterations. Simulations were performed three times to ensure reliable convergence. Fitting distributions and the Monte Carlo simulations were carried out using the software package @Risk for Microsoft Excel version 6 (Palisade Corporation, US). The results were reported as estimated daily exposure of each mycotoxin expressed as ng kg^{-1} bw day^{-1}. Combined dietary exposure assessment was based on the cumulative assessment (CA) concept that consisted in summing the same toxin present in different staple foods (EFSA, 2013).

2.4. Risk characterization

For individual mycotoxins, the estimated daily exposure was compared to the toxicological thresholds of non-carcinogenic mycotoxins, i.e. tolerable daily intake (TDI) or provisional maximum tolerable daily intake (PMTDI): OTA (PMTDI 17 ng kg^{-1} bw day^{-1}) (EFSA, 2010), sum of HT-2 and T-2 toxins (TDI 100 ng kg^{-1} bw day^{-1}) (EFSA, 2011) and DON (TDI 1000 ng kg^{-1} bw day^{-1}) (SCF, 2002). These comparisons were reported as percentages of the population at risk of exceeding the corresponding toxicological threshold. In addition, the Hazard Quotient (HQ), calculated as the ratio between average exposure and the toxicological threshold, was reported. A ratio of HQ > 1 implies a non-tolerable exposure level (Assunção et al., 2015; EFSA, 2013). For carcinogenic mycotoxins, i.e. AFB_1 and AFM_1, the approach of the Margin of Exposure (MOE) was calculated as the ratio between the BMDL (lower confidence limit of the bench mark dose) and the average estimated daily exposure (Pratt et al., 2009). Specifically, the $BMDL_{10}$ (10% extra cancer risk) of 170 ng kg^{-1} bw day^{-1} was used that corresponds to hepatocarcinoma in experimental rats as effect of the exposure to AFB_1 and total aflatoxins (EFSA, 2007).

For combined exposure, the component-based approach based on the CA methods of Hazard Index (HI) and Combined Margin of Exposure Index (MOET) were applied for thresholded and non-thresholded mycotoxins, respectively (EFSA, 2013). The HI was calculated as the sum of the respective HQ's for DON and OTA across wheat noodles and oat flakes. A value of HI > 1 implies non-acceptable level of the total mixture concentration. The MOET was calculated as the reciprocal of the sum of the reciprocals of the individual MOE for aflatoxins in breast milk (EFSA, 2013).

According to the European Food Safety Authority (EFSA) and the World Health Organization (WHO), a MOE for a single substance of 10,000 and above is considered of low concern for public health, and therefore, low priority for risk management actions (EFSA, 2007; Pratt et al., 2009). There are no established criteria to define the magnitude of an acceptable MOE for mixtures of chemicals that are both genotoxic and carcinogenic, such as aflatoxins.

3. Results and discussion

3.1. Occurrence of AFB_1 and AFM_1 in breast milk

The recovery of the analytical method for the analysis of AFB_1 and AFM_1 was 99 ± 6% and 88 ± 4%, respectively. The method yielded an intra- and inter-day precision of 13.1% and 6.8% for AFM_1 and 1.3% and 9.3% for AFB_1, respectively. Both, recovery and precision were in compliance with the regulation 2002/657/EC (European-Commission, 2002). The LOD's and LOQ's for AFM_1 and AFB_1 were 0.033 and 0.066 μg L^{-1}, and 0.023 and 0.046 μg L^{-1}, respectively. Aflatoxin M_1 was quantified in 10 out of 78 breast milk samples (13%) and one sample was within the LOD-LOQ range. The concentration of AFM_1 ranged from 53 to 458 ng L^{-1} (216 ± 116 ng L^{-1}). All breast milk samples exceeded the maximum limit for AFM_1 in infant milk set by the EU regulation (25 ng kg^{-1}) (European-Commission, 2012). Aflatoxin B_1 was quantified in 7 out of 78 breast milk samples (9%) in a concentration range of 56–291 ng L^{-1} (147 ± 89 ng L^{-1}) and no maximum permissible level has been set yet for this mycotoxin in infant milk.

The observed contamination levels of AFM_1 in breast milk were

Table 1
Input contamination data for exposure assessment per food source, feeding pattern category and region, expressed as ng kg^{-1} for staple cereals and ng L^{-1} for breast milk.

	n[a]	n < LOD[b]	Mycotoxin	Mean[c]	SD	Min[d]	Max
Breast milk	78	67/78	AFM_1	45	81	17	458
	78	71/78	AFB_1	24	46	12	291
Polished rice	46	44/46	HT-2	11,172	4869	10,190	39,540
Wheat noodles	80	64/80	DON	48,379	32,641	26,710	224,172
	139	137/139	OTA	4336	8309	950	93,118
Oat flakes	42	35/42	DON	21,253	24,253	13,200	151,545
	77	74/77	OTA	4531	18,194	750	161,570

[a] Number of samples.
[b] Number of samples below the limit of detection and assigned as 0.5 LOD.
[c] Pooled mean of the detected and assigned 0.5 LOD values.
[d] Values corresponding to 0.5 LOD for each combination mycotoxin-matrix (Ortiz et al., 2013a).

similar to the levels reported in some Eastern-European and African countries (El-Tras et al., 2011; Elzupir et al., 2012; Gürbay et al., 2010; Polychronaki et al., 2007), but higher than those reported at different occurrence rates in other LA countries such as in Brazil (2/100 samples; 0.3 and 0.8 ng L^{-1}) (Iha et al., 2014), Mexico (100/112 samples; 3–34 ng L^{-1}) (Cantú-Cornelio et al., 2016) and Colombia (45/50 samples; 0.9–18.5 ng L^{-1}) (Diaz and Sánchez, 2015). From the few available reports of AFB_1 occurrence in breast milk, much higher contamination levels and rate than in this study have been observed in Turkey (75/75 samples; 94.5–4123.8 ng L^{-1}), which contrasts the low contamination level and rate observed in Italy (1/198 samples; 11.4 ng L^{-1}) (Turconi et al., 2004).

3.2. Exposure assessment & risk characterization

The input data for the exposure assessment simulations i.e. mean mycotoxin concentration in medium bound scenario and the best-fit distributions for consumption data are presented in Table 1 and Table 2, respectively.

The estimated daily exposure to mycotoxins (mean, standard deviation and percentiles P50, P75, P90, P95, P97.5 and P99), HQ and/or MOE per dietary source and feeding practice of the consumer child population in the urban and rural area are presented in Table 3 and Table 4, respectively. The HI for the total exposure to DON and OTA through staple cereals intake, and the MOET for the total exposure to aflatoxins through breast milk intake are presented in Table 5.

For the group of youngest children (exclusively or predominantly breastfed), the daily exposure to AFM_1 was slightly higher in the urban (P50-P99: 9.8–30.3 ng kg^{-1} bw day^{-1}) than in the rural area (P50-P99: 9.1–28.6 ng kg^{-1} bw day^{-1}). In lesser extent, the daily exposure to AFB_1 was also slightly higher in the urban area (P50-P99: 5.2–16.1 ng kg^{-1} bw day^{-1}) in comparison to the rural area (P50-P99: 4.8–15.2 ng kg^{-1} bw day^{-1}). Both, MOE and MOET values for these carcinogenic toxins were lower than 10,000 (range 17–122), indicating that these exposures would be of high priority for risk management actions. Those values were substantially lower than the cut-off; however, their interpretation is limited to indicate the need or not of public health concern. Although, since 2012 the EFSA Scientific Committee has recommended the establishment of a more specific risk categorization based on the MOE magnitude (i.e. high concern, low concern or unlikely to be of safety concern), such categorization has not been set yet (EFSA, 2012).

The introduction of complementary foods resulted in a lower average exposure to AFM_1 and AFB_1 through breast milk intake. However, both MOE and MOET values were still far lower than 10,000. The staple cereal-based dietary patterns were similar in both areas at this feeding stage. Polished rice was the most frequently consumed cereal (60% urban and 64% rural), followed by wheat noodles (40% urban and 41% rural) and oat flakes (31% urban and 27% rural). According to the HQ, the general exposure to mycotoxins through staple cereals intake could be considered tolerable. The exposure to HT-2 toxin through rice intake was the most remarkable exposure in the urban area (HQ = 0.42; 4% above TDI; P50-P99: 42.3–131.0 ng kg^{-1} bw day^{-1}) and even more in the rural area where the P99 was about 4 times the TDI (HQ = 0.42; 15% above TDI; P50-P99: 42.4–419.1 ng kg^{-1} bw day^{-1}). Different combinations of staple cereals are consumed by young children in Ecuador. The combined consumption of staple cereals, especially rice, and breast milk was more frequent in the rural area (78%) than in the urban area (55%). Only DON and OTA were present in the other evaluated staple cereals, i.e. wheat noodles oat flakes, and its combined exposures were considered tolerable in both areas (HI < 1).

A substantial increase of the exposure to mycotoxins due to the transition from partial breastfeeding to complete weaning has been

Table 2
Input consumption data for exposure assessment per food source, feeding pattern category and region: number of consumers (n), mean, minimum and maximum and best-fit distribution function in the urban area based on usual daily intake (MSM distribution) and in the rural area based on a single dietary 24-h recall, all expressed as kg kg^{-1} bw day^{-1}.

	Region	n	Mean	Min	Max	Function
Group 1: Exclusively and predominantly breastfed children (urban n = 61; rural n = 72)						
Breast milk	Urban	61	0.2478	0.1004	+ ∞	RiskLoglogistic(0,10037;0,12092;2,9398)
	Rural	72	0.2255	- ∞	+ ∞	RiskExtvalue(0,16485;0,10507)
Group 2: Children at complementary feeding stage (urban n = 303; rural n = 163)						
Breast milk	Urban	277	0.0876	0.0029	+ ∞	RiskInvgauss(0,084706;0,112455;RiskShift(0,0028816))
	Rural	157	0.0925	0.0059	+ ∞	RiskLoglogistic(0,0059066;0,052476;1,9084)
Polished rice	Urban	187	0.0043	−0.0017	+ ∞	RiskInvgauss(0,0059857;0,0365594;RiskShift(-0,0016837))
	Rural	108	0.0059	−0.0002	+ ∞	RiskLoglogistic(-0,00017662;0,0039244;2,0356)
Wheat noodles	Urban	129	0.0015	0.0003	+ ∞	RiskLoglogistic(0,00027747;0,0010397;3,1036)
	Rural	67	0.0013	0.0001	+ ∞	RiskGamma(1,5609;0,00076642;RiskShift(0,00013515))
Oat flakes	Urban	99	0.0007	−0.00006	+ ∞	RiskLognorm(0,00080016;0,00053469;RiskShift(-0,0000673412))
	Rural	42	0.0009	0.0001	+ ∞	RiskExpon(0,0007518;RiskShift(0,00014346))
Group 3: Children at weaning stage (urban n = 239; rural n = 85)						
Polished rice	Urban	194	0.0048	- ∞	+ ∞	RiskExtvalue(0,0037857;0,0018007)
	Rural	73	0.0083	0.0002	+ ∞	RiskLoglogistic(0,00018807;0,0053361;2,0575)
Wheat noodles	Urban	113	0.0015	- ∞	+ ∞	RiskLogistic(0,00145434;0,00027235)
	Rural	46	0.0019	- ∞	+ ∞	RiskExtvalue(0,0012714;0,0010788)
Oat flakes	Urban	121	0.0013	0.0005	+ ∞	RiskPearson5(8,5727;0,013786;RiskShift(-0,0004997))
	Rural	34	0.0014	0.00005	+ ∞	RiskInvgauss(0,0013677;0,0011607;RiskShift(0,0000533386))

Table 3
Estimated daily exposure to mycotoxins per food source for children aged 0–23 months (n = 603) in the urban area categorized according their feeding pattern; percentage of the population exceeding the tolerable daily intake (TDI), hazard quotient (HQ) and margin of exposure (MOE). Means and percentiles are expressed in ng kg^{-1} bw day^{-1}.

	Mean	SD	P50	P75	P90	P95	P97.5	P99	% above TDI[d]	HQ	MOE
Group 1[a]											
AFM_1 in breast milk	11.1	5.2	9.8	12.3	16.1	19.4	23.2	30.3	–	–	17
AFB_1 in breast milk	5.9	2.8	5.2	6.5	8.5	10.3	12.3	16.1	–	–	33
Group 2[b]											
AFM_1 in breast milk	4.0	3.4	3.0	4.9	8.0	10.6	13.4	16.8	–	–	57
AFB_1 in breast milk	2.1	1.8	1.6	2.6	4.2	5.6	7.1	8.9	–	–	108
HT-2 in rice	47.2	26.5	42.3	60.9	82.7	97.9	112.1	131.0	4%	0.42	–
DON in wheat noodles	74.0	45.0	63.7	86.2	116.1	143.8	180.4	236.1	0%	0.06	–
OTA in wheat noodles	6.6	4.0	5.7	7.7	10.4	12.9	16.2	21.2	2%	0.34	–
DON in oat flakes	15.7	11.6	12.7	20.1	29.5	36.6	44.6	57.9	0%	0.01	–
OTA in oat flakes	3.3	2.5	2.7	4.3	6.3	7.8	9.5	12.3	0%	0.16	–
Group 3[c]											
HT-2 in rice	54.0	26.2	49.6	67.4	87.7	102.7	118.5	138.6	6%	0.50	–
DON in wheat noodles	69.9	24.2	69.9	84.6	99.5	109.6	119.1	129.2	0%	0.07	–
OTA in wheat noodles	6.3	2.2	6.3	7.6	8.9	9.8	10.7	11.6	0%	0.37	–
DON in oat flakes	27.8	14.9	24.6	34.3	46.2	56.0	66.2	78.9	0%	0.02	–
OTA in oat flakes	5.9	3.2	5.2	7.3	9.9	11.9	14.1	16.8	1%	0.31	–

[a] Group 1 (n = 61), children being exclusively/predominantly breastfed (only water and water-based drinks, vitamins, minerals and medicines could be consumed besides breast milk). Average children age: 2.6 ± 1.7 months of age (min-max = 0.2–7.6).
[b] Group 2 (n = 303), children at complementary feeding stage (cereal and cereal products & breast milk): rice consumers (n = 187), wheat noodles consumers (n = 129), oat flakes consumers (n = 99) and breast milk consumers (n = 277). Average children age: 10.9 ± 5.6 months of age (min-max = 0.3–23.6).
[c] Group 3 (n = 239), children at weaning stage (no breast milk intake): rice consumers (n = 194), wheat noodles consumers (n = 113) and oat flakes consumers (n = 121). Average children age: 16.3 ± 5.7 months of age (min-max = 0.5–24.0).
[d] PMTDI was used for risk characterization of OTA.

Table 4
Estimated daily exposure to mycotoxins per food source for children aged 0–23 months (n = 320) in the rural area categorized according their feeding pattern; percentage of the population exceeding the tolerable daily intake (TDI), hazard quotient (HQ) and margin of exposure (MOE). Means and percentiles are expressed in ng kg^{-1} bw day^{-1}.

	Mean	SD	P50	P75	P90	P95	P97.5	P99	% above TDI[d]	HQ	MOE
Group 1 [a]											
AFM_1 in breast milk	10.1	6.1	9.1	13.2	18.2	21.6	24.7	28.6	–	–	19
AFB_1 in breast milk	5.3	3.2	4.8	7.0	9.6	11.4	13.1	15.2	–	–	35
Group 2 [b]											
AFM_1 in breast milk	4.1	8.5	2.6	4.4	7.5	11.0	16.0	27.3	–	–	65
AFB_1 in breast milk	2.2	4.5	1.4	2.4	4.0	5.8	8.5	14.5	–	–	122
HT-2 in rice	67.9	177.7	42.4	74.6	128.6	188.2	269.9	419.1	15%	0.42	–
DON in wheat noodles	64.3	45.6	52.8	85.3	125.0	153.3	181.4	216.0	0%	0.05	–
OTA in wheat noodles	5.8	4.1	4.7	7.6	11.2	13.7	16.3	19.4	2%	0.28	–
DON in oat flakes	19.1	15.9	14.2	25.3	39.9	51.2	60.9	76.3	0%	0.01	–
OTA in oat flakes	4.1	3.4	3.0	5.4	8.5	10.9	13.0	16.3	1%	0.18	–
Group 3 [c]											
HT-2 in rice	89.9	157.1	61.7	102.6	168.8	236.4	325.1	528.8	26%	0.62	–
DON in wheat noodles	91.5	66.7	80.2	127.2	177.2	215.5	251.2	301.6	0%	0.08	–
OTA in wheat noodles	8.2	6.0	7.2	11.4	15.9	19.3	22.5	27.0	8%	0.42	–
DON in oat flakes	29.6	30.3	19.5	36.4	63.1	86.7	114.0	149.4	0%	0.02	–
OTA in oat flakes	6.3	6.5	4.2	7.8	13.4	18.5	24.3	31.8	6%	0.24	–

[a] Group 1 (n = 72), children being exclusively/predominantly breastfed (only water and water-based drinks, vitamins, minerals and medicines could be consumed besides breast milk). Average children age: 3.3 ± 2.0 months of age (min-max = 0.3–10.5).
[b] Group 2 (n = 163), children at complementary feeding stage (cereal and cereal products & breast milk): rice consumers (n = 108), wheat noodles consumers (n = 67), oat flakes consumers (n = 42) and breast milk consumers (n = 157). Average children age: 12.8 ± 5.0 months of age (min-max = 1.0–23.9).
[c] Group 3 (n = 85), children at weaning stage (no breast milk intake): rice consumers (n = 73), wheat noodles consumers (n = 46) and oat flakes consumers (n = 34). Average children age: 18.1 ± 5.7 months of age (min-max = 4.3–24.0).
[d] PMTDI was used for risk characterization of OTA.

suggested elsewhere (Gong et al., 2003; Kimanya et al., 2014; Shouman et al., 2012). In this study, at weaning stage polished rice was also the most frequently consumed cereal in both areas (81% urban and 86% rural), followed by the consumption of wheat noodles (49% urban vs. 53% rural) and oats flakes (51% urban vs. 41% rural). The combination of different staple cereals on daily bases was more common in the rural area than in the urban area (68% vs. 44%). The most prevalent combinations were polished rice with wheat noodles (30% rural and 13% urban); with oat flakes (18% rural and 19% urban), and with wheat noodles & oat flakes (16% rural and 7% urban). Since only HT-2 toxin exposure was assessed due to the contamination pattern of Ecuadorian rice (Table 1), this was the most remarkable exposure in both areas considering the significant rice consumption. This exposure was considerably high in the rural area (HQ = 0.62; 26% above TDI; P50-P99: 61.7–528.8 ng kg^{-1} bw day^{-1}) being the P99 about 5 times the TDI. Whereas, the exposure of HT-2 toxin in the urban area (HQ = 0.50; 6% above TDI; P50-P99: 49.6–138.6 ng kg^{-1} bw day^{-1}) was similar to the exposure observed at complementary feeding stage. On the other hand,

Table 5
Combined Margin of Exposure Index (MOET) for the total exposure to aflatoxins through breast milk intake and Hazard Index (HI) for the total exposure to deoxynivalenol (DON) and Ochratoxin A (OTA) through wheat noodles and oat flakes intake, for urban and rural children aged 0–23 months (n = 923) categorized according their feeding pattern.

	MOET		HI	
	Urban	Rural	Urban	Rural
Group 1 [a]				
Aflatoxins in breast milk	10	11	–	–
Group 2 [b]				
Aflatoxins in breast milk	28	27	–	–
DON in wheat noodles and oat flakes	–	–	0.08	0.07
OTA in wheat noodles and oat flakes	–	–	0.50	0.46
Group 3 [c]				
DON in wheat noodles and oat flakes	–	–	0.09	0.10
OTA in wheat noodles and oat flakes	–	–	0.68	0.67

[a] Group 1 (n = 133), children being exclusively/predominantly breastfed (only water and water-based drinks, vitamins, minerals and medicines could be consumed besides breast milk).

[b] Group 2 (n = 466), children at complementary feeding stage (cereal and cereal products & breast milk).

[c] Group 3 (n = 324), children at weaning stage (no breast milk intake).

the combined exposure to OTA and DON at weaning stage reached acceptable HI values in both areas.

Overall, the exposure to mycotoxins through staple cereals was rather modest, except of the remarkable exposure to HT-2 toxin through rice intake. The exposure was higher for rural children due to their monotonous cereal-based diet. In particular, this was evidenced with the increased exposure during the transition from complementary feeding to weaning stage. In contrast, a more varied diet of the urban children may lead to a diluting effect of the overall exposure to mycotoxins or may also lead to the introduction of other dietary sources of mycotoxins, such as dairy products and other cereals. However, this was not assessed in this study as well as the potential presence of modified mycotoxins (Berthiller et al., 2013). On the other hand, the different exposures of rural and urban children could also be related to the age distribution of the feeding categories (Tables 3 and 4). This differed significantly between areas ($P = 0.049$ for group 1; $P = 0.0003$ for group 2; $P = 0.011$ for group 3) and rural child feeding patterns are characterized by longer breastfeeding stages and their subsequent risk of exposure.

Regarding the exposure through breastfeeding, besides aflatoxins, several mycotoxins and their metabolites can potentially transferred to human breast milk (Degen et al., 2013; Ediage et al., 2013; Iha et al., 2014; Muñoz et al., 2010; Navas et al., 2005; Rubert et al., 2014; Turconi et al., 2004). In LA countries, the main focus has been the study of lactational transfer of OTA and AFM_1 as reported in Chilean (Muñoz et al., 2010, 2014) and Brazilian studies (Iha et al., 2014; Navas et al., 2005). Thus, further multimycotoxin analysis of biomarkers might be of first choice to understand the potential risk of nursing infants in the studied region (Ediage et al., 2013; Turner et al., 2012). The pivotal role of breastfeeding in infant nutrition must be preserved; therefore, actions must be taken to improve the quality of maternal diet. To consider, the cereals used for complementary foods are also staple cereals in the usual Ecuadorian diet. Consequently, nursing mothers could be potentially exposed to several mycotoxins. The assessment of combined exposures should be further explored aiming to provide an epidemiological overview of the mycotoxin food hazard in those highly vulnerable population groups (Meek et al., 2011). In addition, some sources of variations in maternal exposure to mycotoxins should be taken into account in further studies, such as regional differences in dietary patterns (Mahdavi et al., 2010) and socio-economic disparities (Peraica et al., 2014). In this regard, the limitation of collecting breast milk samples from only rural mothers might be considered a worse scenario for urban children.

Different approaches to evaluate the exposure to multiple mycotoxins from multiple sources have been proposed (EFSA, 2013). Particularly, the approach of grouping mycotoxins with plausible common mode of action could be of concern in this study. For instance, regarding the suggested effect of immunomodulation of trichothecenes, OTA and aflatoxins, as well as the hypothesized interaction of aflatoxins, fumonisins and DON with childhood growth faltering (Peraica et al., 2014; Smith et al., 2012). Even though, its application is still complex and challenging in terms of modelling of additive risk assessment and biological pathways interpretation when considering synergism and mycotoxin concentrations (Assunção et al., 2016).

The staple cereals evaluated in this study were from different origin, i.e. either locally cultivated (rice), imported as raw material (wheat flour), and imported as final product (oat flakes). Therefore, possible risk management strategies could be driven towards improvement of agricultural and storage practices, as well as enforcement of regulations for imported goods. Due to the high consumption, special attention should be given to potential mycotoxin contamination in polished rice. This should not be only focused on HT-2 toxin, but also on other mycotoxins that might remain after rice milling. On the other hand, maximum permitted levels for the most prevalent mycotoxin-cereal combination for imported and produced goods should be included in Ecuadorian regulations, which are currently very scarce.

3.3. Uncertainties related to the risk assessment

In this study some uncertainties need to be addressed towards a better understanding and interpretation of the results and their implications. First, contamination data of the majority of the cereal samples was attributed as half of the limit of detection due to the very low mycotoxin occurrence (as detailed in Table 1). This could represent a positive biased scenario, suggesting that the exposure severity might be softer. This would happen if the undetected data would be left-skewed as likely given for environmental contaminants distributions that degrade following first-order kinetic, usually adopting a lognormal shape. However, the imputed contamination data were best-fitted as Pareto distributions due to the large number of undetected values. Instead, mean contamination values were used for the exposure assessments. Secondly, seasonal variations could influence the contamination degree (Jonsyn-Ellis, 2001), but it was not considered when sampling staple cereals for multi-mycotoxin analysis. Moreover, a likelihood of worsened contamination patterns due to climate change has been suggested (Tirado et al., 2010; Uyttendaele et al., 2015) and, therefore, current contamination patterns should be assessed in follow-up studies. Another uncertainty source would be that the exposure assessment computation for rural children was carried out based on only one 24-h recall due to logistic constraints. This might lead to the estimation of non-usual intake; however, the statement of monotonous rural diet is based on intake studies (data not shown) and food accessibility in this area. Finally, this study was carried out using contamination data of staple cereals analyzed on raw dry basis. Although, the employment of these kind of data is a commonly accepted methodology (Lambe, 2002), the use of contamination data of foods as consumed could contribute to the reduction of degree of uncertainties. Cereal processing and cooking could reduce mycotoxin content (Stoev, 2013) and this might lead to certain variations in the mycotoxin exposure patterns. For instance, the water-soluble DON can be partially removed due to leaching into the cooking water (Kushiro, 2008). On the other hand, most mycotoxins are heat-stable and hydrophobic; consequently, slight reduction might be expected due to cooking (Bhat et al., 2010; Stoev, 2013).

4. Conclusion

This study is the first report of dietary exposure assessment to

mycotoxins in Ecuadorian children aged 0–23 months through breast milk and staple cereals (polished rice, wheat noodles and oat flakes) used for complementary feeding. The presence of the carcinogenic AFB_1 and its cytotoxic metabolite AFM_1 in breast milk of nursing mothers was evidenced. Moreover, due to their more monotonous cereal-based diet, children from the rural area were considerably more exposed to mycotoxins, such as HT-2 through polished rice intake and in lesser extent to DON and OTA through wheat noodles and oat flakes intake.

In general, we report a modest exposure to mycotoxins, with the exception of the considerable exposure to HT-2 toxin due to the high rice consumption. However, follow-up studies to fill some gaps could be recommended to discard significant exposure to mycotoxins in Ecuadorian young children. In addition, this could represent a public health and food safety challenge considering the potential chronic exposure due to the frequent consumption of those staple foods, as well as the possible additive of synergic adverse effects of multiple mycotoxin exposure even at low levels.

The development of risk management actions for further monitoring and mitigation of mycotoxin contamination in staple cereals, especially for the locally cultivated and highly consumed rice, could drive to the protection of young children as well as general consumers. Of particular importance, the food safety of nursing mothers must be ensured for subsequent reduction of lactational transfer of aflatoxins and other likely mycotoxins. Additionally, a possible shifting to alternative staples cereals as complementary foods could be considered but this might demand assessments, including consumers' accessibility and acceptability evaluations as well as mycotoxin analysis in foods and as biomarkers.

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