*"Una persona usualmente se convierte en aquello que cree que es. Si yo sigo diciéndome a mí mismo que no puedo hacer algo, es posible que termine siendo incapaz de hacerlo. Por el contrario, si yo tengo la creencia que sí puedo hacerlo, con seguridad adquiriré la capacidad de realizarlo aunque no la haya tenido al principio."*

*"Man often becomes what he believes himself to be. If I keep on saying to myself that I cannot do a certain thing, it is possible that I may end by really becoming incapable of doing it. On the contrary, if I have the belief that I can do it, I shall surely acquire the capacity to do it even if I may not have it at the beginning."*

***Mahatma Gandhi***

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# Infant feeding, nutritional status and mycotoxin exposure of children in the Ecuadorian highlands

Silvia Johana Ortiz Ulloa

*Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Applied Biological Sciences: Chemistry*

Duch translation of the title:

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADI | Acceptable Daily Intake |
| AF | Aflatoxin |
| ALOP | Appropriate Level of Protection |
| ATA | Alimentary Toxic Aleukia |
| BMD | Bench Mark Dose |
| BMDL | Low confidence limit of Bench Mark Dose |
| BMI | Body Mass Index |
| BMIZ | BMI-for-Age Z-Score |
| bw | Body Weight |
| CAN | Nations of the Andean Communities |
| CATCH | Rapid Core Assessment Tool on Child Health |
| CI | Confidence Interval |
| DNA | Deoxyribonucleic Acid |
| DON | Deoxynivalenol |
| DRI | Dietary Reference Intake |
| EC | European Commission |
| EFSA | European Food Safety Authority |
| EIC | Extracted Ion Chromatogram |
| ESI | Electrospray Ionization |
| FANTA | Food And Nutrition Technical Assistance |
| FAO | Food And Agriculture Organization |
| $FB_1$ | Fumonisin $B_1$ |
| FDA | Food And Drug Administration |
| FLD | Fluorescence Detector |
| FWHM | Full width at half maximum |
| HPLC | High Performance Liquid Chromatography |
| IARC | International Agency for Research on Cancer |
| INFA | National Institute of Childhood and Family |
| INFOODS | International Network of Food Data Systems |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| KPC | Knowledge Practices Coverage |
| LAZ | Length-for-Age |

| | |
|---|---|
| LC | Liquid Chromatography |
| LOAEL | Lowest Observable Adverse Effect Level |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| MERCOSUR | "Mercado Común Del Sur" or Southern Common Market |
| MGRS | Multicentre Growth Reference Study |
| MMCC | Matrix-Matched Calibration Curve |
| MOE | Margin of Exposure |
| MS | Mass Spectrometry |
| MSM | Multiple Source Method |
| MUAC | Mid-Upper Arm Circumference |
| NC | Non-carcinogenic |
| NOAEL | No Observable Adverse Effect Level |
| NTC | Non-threshold carcinogen (DNA reactive) |
| OR | Odds Ratio |
| ORS | Oral Rehydration Salts |
| OTA | Ochratoxin A |
| PBS | Phosphate-Buffered Saline Solution |
| PCA | Principal Component Analysis |
| PMTDI | Provisional Maximum Tolerable Daily Intake |
| ppm | Parts per million |
| PTDI | Provisional Tolerable Daily Intake |
| RE | Retinol Equivalent |
| RSD | Relative Standard Deviation |
| RT | Retention Time |
| SCF | Scientific Committee on Food |
| SD | Standard Deviation |
| SDp | Standard Deviation of a Sample Proportion |
| SES | Socioeconomic Status |
| SIISE | Integrated Social Indicator System for Ecuador |
| SSE | Signal Suppression-Enhancement |
| TC | Threshold carcinogen (non-DNA reactive) |
| TDI | Tolerable Daily Intake |
| TIC | Total Ion Chromatogram |

| | |
|---|---|
| TOF | Time-of-Flight |
| UBN | Unsatisfied Basic Necessities |
| UHPLC | Ultra High Performance Liquid Chromatography |
| UNA | “Unidad Nacional de Almacenamiento” or National Storage Unit |
| UNICEF | United Nations Children’s Fund |
| USDA | U.S. Department Of Agriculture |
| WAZ | Weight-for-Age |
| WHO | World Health Organization |
| WLZ | Weight-for-Length |
| WWN | White Wheat Noodles |
| YWN | Yellow Wheat Noodles |
| ZAN | Zearalanone |
| ZEN | Zearelenone |

# SUMMARY

## SUMMARY

In developing countries, poverty and food insecurity can compromise child's welfare and particularly child nutritional status. The pathogenesis of child malnutrition is very diverse. Particularly, in the frame of the complex etiology of stunting, associations with mycotoxin exposure have been suggested.

The scope of the present study was the evaluation of the nutritional status and the associations of child malnutrition with socio-demographic characteristics; the evaluation of child feeding patterns and the assessment of dietary exposure to mycotoxins at early childhood in the Ecuadorian highlands.

This study was based on a cross-sectional survey conducted in 2008. The participants were children aged 0-23 months (n=998) and their caregivers from a rural canton (Nabon) and an urban canton (Cuenca), Azuay province. Data on anthropometric measurements, socio-demographic characteristics, feeding practices, food intake (24 hours recall) and childcare practices were collected. Child nutritional status was assessed and socio-demographic determinants of stunting, wasting and overweight were identified and compared between both areas. Feeding patterns, i.e. food intake and feeding practices were described and the difference between urban and rural areas was evaluated. The assessment of child feeding practices was based on the indicators recommended by the World Health Organization (WHO). The main cereal-based staple foods were identified for subsequent evaluation of mycotoxin contamination. Two food sampling plans were designed: one for retail products (collected in Cuenca and Nabon) and another for products in bulk (collected at the coastal region of the country). The staple cereals collected for multimycotoxin analysis were rice (paddy and polished), wheat noodles (white and yellow) and oat flakes. In addition, the contamination of breast milk with aflatoxin $M_1$ was assessed. Finally, the risk of exposure to mycotoxins through the intake of cereal staple foods and breast milk was evaluated. The exposure assessment was performed using a simple distribution method and first order Monte Carlo simulation.

The prevalence of stunting was significantly higher in the rural area (37.4% vs. 17.7%, $P<0.001$). The prevalence of wasting was rather low in both areas (7.1%). The results suggested a double burden of malnutrition, i.e. stunting and overweight. Considerable prevalence of overweight was observed in the urban area, however it was not significantly higher than the prevalence in the rural area (12.4% vs. 8.8%, $P=0.309$). Maternal nutritional status was a determinant for stunting in both areas, i.e. maternal BMI in the urban area (OR=0.91; 95% CI: 0.84, 0.99; $P=0.027$) and maternal height in the rural area (OR=0.004; 95% CI: 0.00004, 0.39; $P=0.018$). Moreover, diarrhea prevalence (OR=2.18; 95% CI: 1.13, 4.21; $P=0.020$), socio-economic status (SES) (OR=0.79; 95% CI: 0.64, 0.98; $P=0.030$) and child's age (OR=1.07; 95% CI: 1.02, 1.11; $P=0.005$) were also rural determinants for stunting. No rural determinants for

wasting or overweight were observed. In the urban area, facility-based delivery (OR=0.25; 95% CI: 0.09, 0.73; *P*=0.011) and prevalence of cough (OR=0.57; 95% CI: 0.34, 0.96; *P*=0.036) were determinants for overweight, and hygienic practices for wasting (OR=0.57; 95% CI: 0.36, 0.89; *P*=0.013).

Child feeding practices were different between areas. In general, breastfeeding practices were better in the rural area, whereas in the urban area a higher intake of solid/semi-solid foods at complementary feeding (when children were still breastfed) and at weaning feeding (when children were not longer breastfed) was observed. This child's diet in both settings was mainly based on cereals and cereal products (20% urban vs. 26% rural), dairy products (29% urban vs. 18% rural), fruits and vegetables (11% urban vs. 15% rural) and tubers (11% urban vs. 22% rural). In both areas, the consumption of plant-sources of vitamin A and flesh foods (meat, poultry and liver/organ meats) was remarkably low. Non-timely initiation of complementary feeding (50% vs. 20%, *P*<0.001) and weaning (10% vs. 4%, *P*<0.001) in infants younger than 6 months was higher in the urban area. Facility-based delivery was a determinant for exclusive breastfeeding (OR=2.52; 95% CI: 1.22, 5.21; *P*=0.012) and for timely introduction of solid/semi-solid foods (6-8 months) (OR=9.93; 95% CI: 1.53, 64.36; *P*=0.016). Facility-based delivery was positively associated with average energy intake at complementary feeding stage (56.3 kcal; 95% CI: 5.65, 107; *P*=0.030) and with the energy intake of solid/semi-solid foods (114 kcal; 95% CI: 80.3, 148; *P*<0.001). On the other hand, facility-based delivery was negatively associated with the total duration of breastfeeding (-1.36 months; 95% CI: -2.59, -0.13; *P*=0.030) and the age of introduction of solid/semi-solid foods (-0.46 months; 95% CI: -0.78, -0.14; *P*=0.005). Single-parent households were a determinant of "ever" breastfeeding (OR=0.26; 95% CI: 0.12, 0.54; *P*<0.001) and it was negatively associated with the age of introduction of solid/semi-solid foods (-0.40 months; 95% CI: -0.76, -0.04; *P*=0.028). "Ever" breastfeeding (OR=0.39; 95% CI: 0.18, 0.86; *P*=0.020) and continued breastfeeding at 2 years (OR=0.20; 95% CI: 0.08, 0.53; *P*=0.001) were less likely in households that received additional income from migration. Larger household size (households with more children under 5 years) was a determinant of breastfeeding within the first hour after birth (OR=1.52; 95% CI: 1.06, 2.17; *P*=0.023) and was positively associated with the average energy intake from breast milk (33.4 kcal; 95% CI: 4.01, 62.8; *P*=0.026) and from solid/semi-solid foods (28.5 kcal; 95% CI: 1.87, 55.1; *P*=0.036). Maternal work outside home was positively associated with the average energy intake at complementary feeding stage (57.1 kcal; 95% CI: 20.2, 94; *P*=0.003) and the average energy intake from solid/semi-solid foods (31.0 kcal; 95% CI: 1.16, 60.9; *P*=0.042). Maternal education level was a determinant for timely introduction of solid/semi-solid foods (6-8 months) (OR=1.28; 95% CI: 1.14, 1.43; *P*<0.001) and it was also positively associated with the average energy intake from solid/semi-solid foods (10.8 kcal; 95% CI: 6.04, 15.6; *P*<0.001).

The differences in average energy and nutrient intake between the urban and rural

setting were assessed by adjusting regression models for facility-based delivery; health seeking behavior, maternal age, maternal age at first delivery, maternal education, maternal working status, number of children under 5 years at the household, maternal marital status and additional income from migration were added to the analytical models. The urban-rural difference in breast milk intake remained after adjustment, while those covariates partially explained the urban-rural difference in energy intake from solid/semi-solid foods (52-66%) and in feeding indicators (9-44%). Maternal education and facility-based delivery were the most important socio-demographic covariates that explained the urban-rural differences. Both were defining characteristics in the urban area where the prevalence were significantly higher than in the rural area. This could rely on higher health consciousness and indirectly with better-off household conditions.

The most important cereal staple foods identified in the child's diet were polished rice, oat flakes and wheat noodles. The co-occurrence of aflatoxin $B_1$ ($AFB_1$), $B_2$ ($AFB_2$), $G_1$ ($AFG_1$) and $G_2$ ($AFG_2$), ochratoxin A (OTA), deoxynivalenol (DON), fumonisin $B_1$ ($FB_1$), zearalenone (ZEN), and HT-2 and T-2 toxin was evaluated in representative samples of those cereals. The analytical method using ultra high performance liquid chromatography/time of flight mass spectrometry (UHPLC/TOFMS) was developed and validated for multimycotoxin screening in those cereals and cereal products. Matrix-matched calibration curves were used for quantification and to compensate ion suppression and extraction losses. Most recovery values were in agreement with the minimum requirements of the regulation 401/2006/EC (70-110%). For most mycotoxins, the limits of detection (LOD's) obtained allowed detection in compliance with the maximum permitted levels set in the regulation EC/2006/1881, with exception of OTA in all cereals and $AFB_1$ in yellow wheat noodles. HPLC with fluorescence detection was used for extra target analysis of OTA in oat flakes and wheat noodles, and for the analysis of aflatoxin $M_1$ in breast milk. Mycotoxin occurrence and levels of contamination were rather low. Polished rice was contaminated with $AFG_1$ (2%; 2 µg $kg^{-1}$) and HT-2 toxin (4%; 32.8 ± 9.6 µg $kg^{-1}$). Yellow wheat noodles were contaminated with DON (5%; 85.6 ± 1.9 µg $kg^{-1}$). White noodles were contaminated with DON (33%; 104.4 ± 41.4 µg $kg^{-1}$) and OTA (5%; 60.8 ± 45.7 µg $kg^{-1}$). Oat flakes were contaminated with DON (17%; 61.5 ± 41.8 µg $kg^{-1}$), OTA (4%; 56.1 ± 91.3 µg $kg^{-1}$) and $AFB_1$ (2%; 2.7 µg $kg^{-1}$). Paddy rice was analyzed in order to assess pre-milling conditions. Higher mycotoxin occurrence was observed in paddy rice, which was contaminated with DON (23%, 76.4 ± 20.3 µg $kg^{-1}$), $FB_1$ (23%, 206 ± 345.4 µg $kg^{-1}$), $AFB_1$ (7%, 20.6 ± 23.3 µg $kg^{-1}$), $AFG_1$ (2%, 63.7 µg $kg^{-1}$) and $AFG_2$ (2%, 3.3 µg $kg^{-1}$), Mycotoxin co-occurrence was observed only in white wheat noodles (5%, combinations of DON and OTA) and paddy rice (7%, combinations of $AFG_1$, $AFB_1$, DON and $FB_1$). Breast milk was contaminated with $AFM_1$ (12%; 0.032 ± 0.016 µg $L^{-1}$).

The exposure to mycotoxins through multiple food sources differed according the child feeding stage (exclusive and predominant breastfeeding, complementary feeding and

weaning). The exposure to $AFM_1$ through the consumption of breast milk in exclusively/predominantly breastfed children was high in both areas. Similar margin of exposure (MOE) values (153 urban vs. 168 rural) were observed in both rural and urban area; as well as similar proportion of the population above the PMTDI (provisional maximum tolerable daily intake) (49% urban vs. 44% rural) and the P99 was 3 times above the PMTDI. In children at complementary feeding stage, the most important health risk was the exposure to HT-2 through polished rice. The proportion of the population above the TDI was considerably higher in the rural area (15% vs. 5%) as well as the P99 (4 vs. 1.3 times above the TDI). At weaning stage, rural children were higher exposed to mycotoxins due to the higher consumption of cereal staple foods. The most important health risk was the exposure to HT-2 through polished rice (26%; P99 exceeding 5.5 times the TDI) and OTA through wheat noodles (15%; P99 exceeding 2 times the PTDI) and oat flakes (10%; P99 exceeding 2.4 times the PTDI).

The outcomes of this study could be used as bases for nutrition-sensitive interventions, i.e. community-based intervention strategies in which different underlying causes of child malnutrition can be covered and food safety aspects could be integrated. Intervention strategies could also involve agricultural (pre- and post-harvest) and decontamination or chemical detoxification approaches. Agronomical efforts could be oriented to risk management in particular for polished rice. Besides intervention strategies, risk communication at policy level was encouraged since no mycotoxin regulations are enforced in Ecuador.

## SAMENVATTING

In ontwikkelingslanden wordt het welzijn en meer in het bijzonder de voedingsstatus van kinderen vaak verstoord door armoede en voedselonzekerheid. De pathogenese van voedingsstoornissen bij kinderen is echter heel divers. In de context van groei-achterstand (stunting), werd een verband gesuggereerd met de blootstelling aan mycotoxinen.

In dit onderzoek werd de voedingsstatus van kinderen in het hooggebergte van Ecuador onderzocht, alsook de associaties tussen malnutritie en socio-demografische kenmerken, de evaluatie van het voedingspatroon en de blootstelling aan mycotoxinen bij jonge kinderen.

Deze studie is gebaseerd op een cross-sectioneel onderzoek uitgevoerd in 2008 in de provincie Azuay, Ecuador. De deelnemers waren kinderen in de leeftijdscategorie 0-23 maand (n=998) samen met hun zorgverstrekkers, die wonen in een ruraal gebied (Nabon) en in een verstedelijkt gebied (Cuenca). Anthropometrische data, socio-demografische kenmerken, voedingspatronen, voedselinname (24 uur recall) en praktijken van kinderverzorging werden verzameld. De voedingsstatus van de kinderen werd bepaald en de diverse socio-demografische determinanten van groei-achterstand (stunting), wasting en overgewicht werden geïdentificeerd en vergeleken in beide zones. Voedingspatronen, meer bepaald voedselinname en voedingsgewoonten, werden beschreven en het verschil tussen het verstedelijkte gebied en de rurale zone geëvalueerd. De voedingsgewoonten van de kinderen werd vergeleken met de wereldgezondheidsindicatoren. De belangrijkste graanproducten werden geïdentificeerd en geselecteerd voor bepaling van mycotoxine contaminatie. Twee staalname plannen werden ontworpen: één voor retail producten (verzameld in Cuenca en Nabon) en een tweede voor bulk producten (verzameld in het kustgebied van Ecuador). De gekozen granen voor mycotoxine analyse waren rijst (paddy en gepolijst), tarwe noodles (wit en geel) en havervlokken. Daarnaast werd ook de contaminatie van moedermelk met aflatoxine $M_1$ onderzocht. Tot slot werd het risico voor blootstelling aan mycotoxinen via de inname aan granen en moedermelk onderzocht. De blootstelling werd onderzocht met een enkelvoudige distributie en met een eerste orde Monte Carlo simulatie.

De prevalentie van groei-achterstand (stunting) was significant hoger in de rurale zone (37.4% vs. 17.7%, $P<0.001$), terwijl wasting tamelijk beperkt bleef in beide zones (7.1%). Deze resultaten wijzen op een dubbele belasting van malnutritie, nl. groei-achterstand en overgewicht. Overgewicht werd duidelijk vastgesteld in de verstedelijkte zone, al was deze niet significant hoger in vergelijking met de rurale zone (12.4% vs. 8.8%; $P=0.309$). De voedingsstatus van het kind was een determinant voor groei-achterstand in zowel de rurale als de verstedelijkte zone, nl. BMI van de moeder in Cuenca (OR=0.91; 95% CI: 0.84, 0.99; $P=0.027$) en lengte van de moeder in Nabon (OR=0.004; 95% CI: 0.00004, 0.39; $P=0.018$). Daarnaast werden ook extra

determinanten voor groei-achterstand geïdentificeerd, nl. prevalentie van diarrhee (OR=2.18; 95% CI: 1.13, 4.21; *P*=0.020), socio-economische status (SES) (OR=0.79; 95% CI: 0.64, 0.98; *P*=0.030) en leeftijd van het kind (OR=1.07; 95% CI: 1.02, 1.11; *P*=0.005). In Nabon werden geen determinanten voor wasting en overgewicht gevonden. In het verstedelijkt gebied waren beschikbare faciliteiten ter ondersteuning van de geboorte (OR=0.25; 95% CI: 0.09, 0.73; *P*=0.011) en prevalentie van hoesten (OR=0.57; 95% CI: 0.34, 0.96; *P*=0.036) determinanten voor overgewicht, en hygiënische omstandigheden was een determinant voor wasting (OR=0.57; 95% CI: 0.36, 0.89; *P*=0.013).

De voedingspatronen van de kinderen in Nabon en Cuenca waren verschillend. Algemeen werden betere praktijken voor borstvoeding vastgesteld in Nabon, terwijl in Cuenca meer vaste en semi-vaste producten werden ingenomen als aanvullende voeding in aan- en afwezigheid van borstvoeding. Het voedingspatroon van het kind in beide regio's was voornamelijk samengesteld uit graan en graanproducten (20% in Cuenca vs. 26% in Nabon), zuivelproducten (29% in Cuenca vs. 18% in Nabon), fruit en groenten (11% in Cuenca vs. 15% in Nabon) en knollen (11% in Cuenca vs. 22% in Nabon). In beide zones was de consumptie van plantaardige vitamin A rijke producten en vleesproducten (vlees, gevogelte en lever/orgaanproducten) merkwaardig genoeg zeer laag. De initiatie van aanvullende voeding (50% vs. 20%, *P*<0.001) en weaning (10% vs. 4%, *P*<0.001) in zuigelingen jonger dan 6 maand was hoger in het verstedelijkt gebied. Faciliteiten ter ondersteuning van de geboorte was een determinant voor exclusieve borstvoeding (OR=2.52; 95% CI: 1.22, 5.21; *P*=0.012) en voor de introductie van vaste en semi-vaste voiding (6-8 maanden) (OR=9.93; 95% CI: 1.53, 64.36; *P*=0.016). Faciliteiten ter ondersteuning van de geboorte werd positief geassocieerd met de gemiddelde energie-inname tijdens complementaire voeding (56.3 kcal; 95% CI: 5.65, 107; *P*=0.030) en met de energie-inname van vaste en semi-vaste voeding (114 kcal; 95% CI: 80.3, 148; *P*<0.001). Daarnaast was de faciliteit ter ondersteuning van de geboorte negatief geassocieerd met de totale duur van borstvoeding (-1.36 maand; 95% CI: -2.59, -0.13; *P*=0.030) en met de leeftijd voor introductie van vaste en semi-vaste voeding (-0.46 maand; 95% CI: -0.78, -0.14; *P*=0.005). Eenoudergezinnen waren een determinant voor het eens voorkomen van borstvoeding (OR=0.26; 95% CI: 0.12, 0.54; *P*<0.001), waarbij een negatieve associatie werd vastgesteld met de leeftijd waarop de vaste en semi-vaste voedingsmiddelen in de voeding worden geïntroduceerd (-0.40 maand; 95% CI: -0.76, -0.04; *P*=0.028). Eens voorkomen van borstvoeding (OR=0.39; 95% CI: 0.18, 0.86; *P*=0.020) en doorgaan met borstvoeding na de leeftijd van 2 jaar (OR=0.20; 95% CI: 0.08, 0.53; *P*=0.001) was minder frequent in huisgezinnen met hogere inkomens door migratie. Grotere huisgezinnen (met meer kinderen jonger dan 5 jaar) was een determinant voor borstvoeding in het eerste uur na de geboorte (OR=1.52; 95% CI: 1.06, 2.17; *P*=0.023) en was positief geassocieerd met de gemiddelde energie-inname uit moedermelk (33.4 kcal; 95% CI: 4.01, 62.8; *P*=0.026) en uit vaste en semi-vaste voeding (28.5 kcal; 95% CI: 1.87, 55.1; *P*=0.036). Uit huis werkende moeders was positief gecorreleerd met de

gemiddelde energie-inname in de periode van aanvullende voeding (57.1 kcal; 95% CI: 20.2, 94; $P$=0.003) en met de gemiddelde energie-inname uit vaste en semi-vaste voeding (31.0 kcal; 95% CI: 1.16, 60.9; $P$=0.042). De opleiding van de moeder was een determinant voor het tijdstip waarop vaste en semi-vaste voeding wordt geïntroduceerd (6-8 maand) (OR=1.28; 95% CI: 1.14, 1.43; $P$<0.001) en was ook positief gecorreleerd met de gemiddelde energie-inname uit vaste en semi-vaste voeding (10.8 kcal; 95% CI: 6.04, 15.6; $P$<0.001).

De verschillen in gemiddelde energie en nutriënt inname tussen Cuenca en Nabon werden onderzocht via aangepaste regressiemodellen voor faciliteiten ter ondersteuning van de geboorte. Gezonde leefstijl, leeftijd van de moeder, leeftijd van de moeder bij de eerste geboorte, opleiding van de moeder, beroep van de moeder, aantal kinderen jonger dan 5 jaar in het huisgezin, burgelijke stand van de moeder, en hoger inkomen door migratie werden toegevoegd aan de analytische modellen. Het verschil in moedermelk inname tussen Cuenca en Nabon bleef behouden na aanpassing, terwijl deze co-variabelen gedeeltelijk het verschil in energie-inname uit vaste en semi-vaste voeding (52-66%) en in voedselinname (9-44%) verklaren. De opleiding van de moeder en de faciliteiten tijdens de geboorte waren de meest belangrijke socio-demografische co-variabelen om het verschil tussen Cuenca en Nabon te verklaren. Beide co-variabelen wijzen op kenmerken van het verstedelijkt gebied waar het voorkomen significant groter was in vergelijking met de rurale zone. Dit kan gebaseerd zijn op meer inzicht en aandacht voor gezondheid in Cuenca en indirect ook met betere leefomstandigheden voor de huisgezinnen.

De meest belangrijke graanproducten in de voeding van de kinderen waren gepolijste rijst, havervlokken en tarwe noodles. Het samen voorkomen van aflatoxine $B_1$ ($AFB_1$), $B_2$ ($AFB_2$), $G_1$ ($AFG_1$) and $G_2$ ($AFG_2$), ochratoxine A (OTA), deoxynivalenol (DON), fumonisine $B_1$ ($FB_1$), zearalenone (ZEN), en HT-2 and T-2 toxine werd onderzocht in representatieve stalen van deze graanproducten. Hiervoor werd een UHPLC/TOFMS analytische methode ontwikkeld en gevalideerd voor multi-mycotoxine screening. Kalibratiecurven aangepast per type matrix warden gebruikt voor kwantificatie en om te compenseren voor ion suppressie en extractieverliezen. De meeste recoveries waren in overeenstemming met de minimale aanbevelingen beschreven in de norm 401/2006/EC (70-110%). Voor de meeste mycotoxinen werden bepalingslimieten (LOD's) bekomen die toelaten de maximaal toelaatbare gehalten in de norm EC/2006/1881 te detecteren, met uitzondering van OTA in alle graanproducten en $AFB_1$ in gele tarwe noodles. HPLC met fluorescentie detectie werd gebruikt voor een extra target analyse van OTA in havervlokken en tarwe noodles, en voor de analysis van aflatoxine $M_1$ in moedermelk.

Het voorkomen van mycotoxinen en de gehalten aan contaminatie was tamelijk laag. Gepolijste rijst was gecontamineerd met $AFG_1$ (2%; 2 µg $kg^{-1}$) en HT-2 toxine (4%; 32.8 ± 9.6 µg $kg^{-1}$). Gele tarwe noodles waren gecontamineerd met DON (5%; 85.6 ±

1.9 µg $kg^{-1}$). Witte noodles waren gecontamineerd met DON (33%; 104.4 ± 41.4 µg $kg^{-1}$) en OTA (5%; 60.8 ± 45.7 µg $kg^{-1}$). Havervlokken waren gecontamineerd met DON (17%; 61.5 ± 41.8 µg $kg^{-1}$), OTA (4%; 56.1 ± 91.3 µg $kg^{-1}$) and $AFB_1$ (2%; 2.7 µg $kg^{-1}$). Paddy rijst werd geanalyseerd om de contaminatie voor het malen na te gaan. Een hogere mycotoxine contaminatie werd gemeten in paddy rijst, meer in het bijzonder voor DON (23%, 76.4 ± 20.3 µg $kg^{-1}$), $FB_1$ (23%, 206 ± 345.4 µg $kg^{-1}$), $AFB_1$ (7%, 20.6 ± 23.3 µg $kg^{-1}$), $AFG_1$ (2%, 63.7 µg $kg^{-1}$) and $AFG_2$ (2%, 3.3 µg $kg^{-1}$). Contaminatie met meerdere mycotoxinen werd enkel vastgesteld voor witte tarwe noodels (5%, combinaties DON en OTA) en paddy rijst (7%, combinaties met $AFG_1$, $AFB_1$, DON en $FB_1$). Moedermelk was gecontamineerd met $AFM_1$ (12%; 0.032 ± 0.016 µg $L^{-1}$).

De blootstelling aan mycotoxinen via meerdere voedingsmiddelen was verschillend alnaargelang de voedingsfase (exclusief/voornamelijk borstgevoed, aanvullende voeding). De blootstelling aan $AFM_1$ via de consumptie van moedermelk in (exclusief/hoofdzakelijk) borstgevoede kinderen was aanzienlijk in zowel Cuenca als Nabon. In zowel de rurale als de verstedelijkte zone werden gelijkaardige resultaten bekomen voor de blootstellings-marges (MOE waarden 153 in Cuenca vs. 168 in Nabon), alsook voor het aandeel van de populatie met innames boven de PMTDI (provisional maximum tolerable daily intake) (49% in Cuenca vs. 44% in Nabon). De P99 was 3 maal hoger dan de PMTDI. In kinderen die aanvullende voeding krijgen, is blootstelling aan HT-2 via gepolijste rijst het belangrijkste gezondheidsrisico. Het aandeel van de bevolking met waarden boven de TDI was significant hoger in de rurale zone (15% vs. 5%). Eenzelfde trend werd gevonden voor P99 (4 vs. 1,3 maal boven de TDI). In de fase van aanvullende voeding zonder extra inname van moedermelk, worden kinderen in de rurale zone meer blootgesteld aan mycotoxinen omwille van de hogere consumptie aan graanproducten. Het voornaamste gezondheidsrisico was de blootstelling aan HT-2 via gepolijste rijst (26%; P99 die 5,5 maal hoger is dan de TDI) en aan OTA via tarwe noodles (15%; P99 2 maal hoger dan de PTDI) en havervlokken (10%; P99 die 2,4 maal hoger is dan de PTDI).

De resultaten uit deze studie kunnen worden gebruikt om gerichte voedingsinterventies te ontwerpen, bv. gemeenschapsinterventies waarin de onderliggende oorzaken van malnutritie bij kinderen worden aangepakt en waarin tevens diverse elementen van voedselveiligheid werden opgenomen. Interventiestrategieën zijn: (1) landbouwkundige interventies (pre- of post-harvest) en (2) decontaminatie of chemische detoxificatie. In het bijzonder kunnen landbouw interventiestrategieën zich richten naar risico-analyse en -beheer van gepolijste rijst. Naast interventiestrategieën wordt ook aanbevolen een gepaste risico-communicatie te voeren op overheidsniveau, aangezien momenteel in Ecuador geen specifieke regelgeving van kracht is mbt mycotoxine contaminatie.

# *INTRODUCTION*

# INTRODUCTION

The interrelation of poverty with health outcomes such as child malnutrition has been widely recognized in developing countries (Van de Poel *et al.*, 2008). The likelihood of poor health and child malnutrition is dependent on environmental and socio-economic conditions that usually differ between rural and urban areas (Smith *et al.*, 2005). Moreover, food security, and subsequently food safety, plays a substantial role on the onset of child malnutrition. In particular, the dietary exposure to secondary fungal metabolites, mycotoxins, has been recognized as an important causal factor of stunting (Shephard, 2008b).

Ecuador is a country located at the northwest of South America that alike Bolivia, Colombia and Paraguay is considered as one of the least developed countries in this region (United-Nations-Development-Programme, 2013). In 2010, according to the survey of Unsatisfied Basic Necessities 60% of the Ecuadorian population was classified as poor (Villacis and Carrillo, 2012). Thus, poverty is one of the main public health problems faced in this country, and together with social-demographic outcomes from migration and urbanization phenomenon, contribute enormously to the deleterious child nutritional situation (Ruel, 2000; Smith *et al.*, 2005; World-Bank, 2007). Ecuador is also part of the small group of Latin American countries with persisting high rates of child malnutrition (World-Bank, 2007). In 2004 national rates of 23% of stunting, 6% of severe stunting and 3% of overweight for children under 5 years old were reported (World-Bank, 2007).

Ecuador has a rich and complex culture making that its population solves their problems of health, production, feeding and housing in different ways. A significant proportion of the indigenous population is concentrated in the highlands, particularly in the rural areas, where traditional beliefs and behaviors might lead to inadequate child feeding and health care practices (World-Bank, 2007). On the other hand, the unique climatologic diversity caused by the presence of the Andes mountain range and the coastal setting offers favorable conditions for fungi development and further production of diverse mycotoxins, which can cause a food safety concern for the local population (Pacin *et al.*, 2002). The exposure to co-occurring mycotoxins could lead to additive or synergic adverse health effects and is particularly critical in staple foods that constitute the bases of the usual monotonous diet of Ecuadorian children.

In Ecuador, very limited published data are available about food safety and nutritional aspects of infants and young children. This PhD thesis describes an explorative study aiming to fill those gaps. The following objectives were formulated:

i) To present a literature review on child malnutrition, socio-demographic constraints, child feeding patterns, mycotoxin contamination in foodstuff and the

dietary exposure to mycotoxins at early life (*Chapter 1*)

ii) To evaluate the nutritional status of children aged 0-23 months from a rural (Nabon) and urban (Cuenca) canton located at the South Ecuadorian highlands, followed by the identification and comparison of the socio-economic, health-related, hygiene and nutritional determinants of stunting, wasting and overweight (*Chapter 2*)

iii) To evaluate the feeding patterns (food intake & feeding indicators) of the same population, identifying the key nutrient sources amongst the staple foods of both areas, and identifying the socio-demographic covariates involved in the urban-rural differences of child feeding patterns (*Chapter 3*)

iv) To determine the occurrence of ten important mycotoxins of health concern (aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A, deoxynivalenol, fumonisin B1, zearalenone, HT-2 and T-2 toxins) in the main Ecuadorian staple cereals used at complementary and weaning feeding stage (rice, oat flakes and white and yellow wheat noodles) (*Chapter 4*)

v) To assess the exposure to mycotoxins of this population through the intake of cereal-based complementary and weaning staple foods and breast milk, establishing oral chronic exposures in order to prioritize risk management strategies (Chapter 5)

Finally, a general discussion, conclusions and perspectives of this study are presented (*Chapter 6*). The outline of this PhD study is schematized in Figure 0.1.

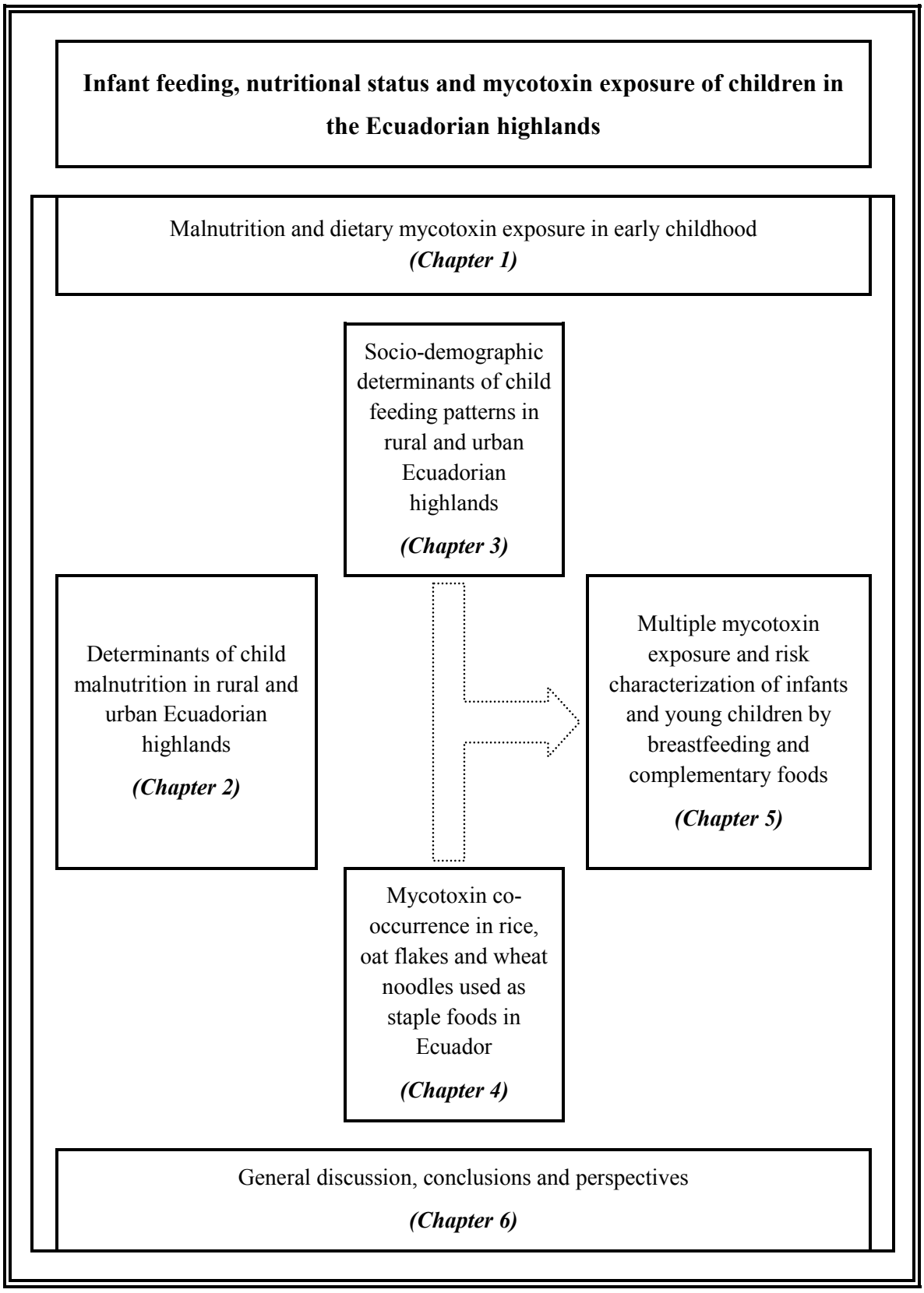



**Figure 0.1.** Schematic outline of the PhD study.

# CHAPTER 1

## *Literature review*

Malnutrition and dietary mycotoxin exposure in early childhood

# 1. Malnutrition and dietary mycotoxin exposure in early childhood

## 1.1. Child malnutrition

### 1.1.1. Food security, child malnutrition and interrelated factors

"Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life" (FAO, 2003).

The complexity of this concept involves several dimensions in which food availability, food access, and food utilization are interrelated with socio-economic factors such as poverty, ecological constraints, inappropriate policies, diseases, poor water and sanitation and inadequate nutritional knowledge (Riely *et al.*, 1999). It has been stated that nutritional status at population level is a manifestation of food utilization, which is determined by the quantity and quality of dietary intake, child feeding practices, general child care including health services, health status and its determinants (Riely *et al.*, 1999). In fact, the multi-causality of child malnutrition has been categorized into four different levels in a proposed modified UNICEF conceptual framework (Figure 1.1), namely temporal, immediate, underlying, and basic level. Maternal nutritional status and fetal growth are considered temporal causes; while immediate causes of malnutrition are related with inadequate dietary intake and poor health status. The underlying causes deal with household food security factors; maternal and childcare; and health, water and sanitation services. Finally, the basic causes are related with cultural and traditional beliefs, moral and ethical principles, education, environmental influences, and infrastructure (Shrimpton and Kachondham, 2003).

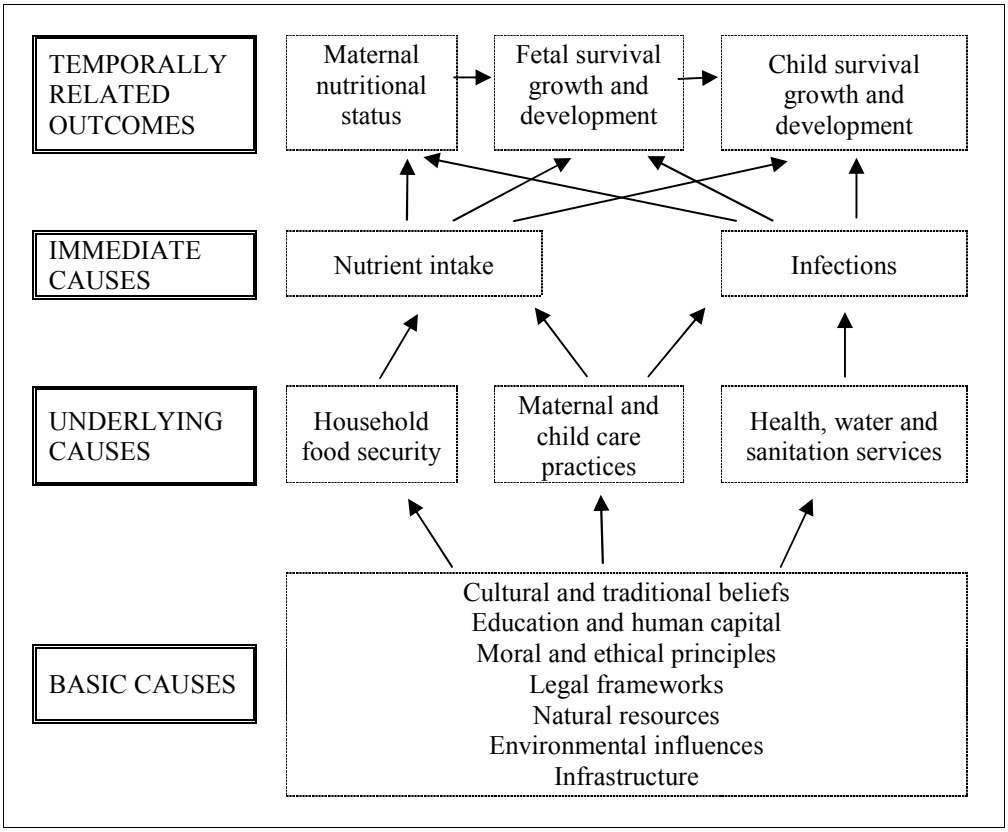



**Figure 1.1.** Modified Conceptual Framework of Child Malnutrition (Source: Shrimptom & Kachondham, 2003) (Shrimpton and Kachondham, 2003).

### 1.1.2. Child malnutrition in Latin America and Ecuador

Globally, maternal and child undernutrition is the underlying cause of 3.1 million deaths and 45% of all child deaths (Black *et al.*, 2013). Fetal growth restriction, stunting, wasting, and deficiencies of vitamin A and zinc along with suboptimum breastfeeding are considered the main health-related problems of child undernutrition. Maternal undernutrition contributes to fetal growth restriction, increasing the rates of stunting by 2 years of age (Black *et al.*, 2013).

Child malnutrition encompasses undernutrition and overweight. Both contribute to important consequences for survival, incidence of acute and chronic diseases, development, and individual and social endowments (Black *et al.*, 2013). The so-called "double burden of malnutrition" i.e. coexistence of growth faltering and/or micronutrient deficiencies with overweight represents the middle stage of nutrition transition, i.e. the shift from undernutrition to overweight and obesity (Chaparro and Estrada, 2012). Nowadays, the double burden of malnutrition is considered as an increasing public health problem in developing countries and can be present at community level, within the same household and even in the same individual at different stages of the life (Black *et al.*, 2013) (Prentice, 2006; Corvalan *et al.*, 2009;

Bove *et al.*, 2012; Chaparro and Estrada, 2012).

In Latin America, child malnutrition remains the major public health problem and this has a strong influence on human development outcomes (Muller and Krawinkel, 2005; Black *et al.*, 2008). In the last decade, rates of nutrition transition have rapidly increased in Latin America, being more pronounced in the urban areas (Prentice, 2006; Chaparro and Estrada, 2012). During the same period, the called "urbanization phenomenon" has turned Latin American cities and metropolitan regions into a combination of high inequality and low social mobility. In this context, South American countries are the most urbanized countries in Latin America (82%) and in those countries a polarized model of nutrition transition among young children has been described. Moreover, besides urbanization, both nutrition transition and child malnutrition are highly influenced by the socio-demographic and socio-economic inequities between rural and urban areas (Ruel, 2000; Smith *et al.*, 2005; Lutter, 2012; Thibert and Osorio, 2013).

Stunting is the most prevalent growth failure in Latin America and it affects children from poor households far more than those from higher socio-economic level (Lutter, 2012). Strong socioeconomic disparities are the common characteristic among Bolivia, Ecuador, and Peru. In these Andean countries, similar high stunting prevalence especially in highland areas and among indigenous populations has been described (Larrea and Freire, 2002; Urke *et al.*, 2011). In contrast, smaller regional disparities and lower stunting prevalence has been observed in Colombia (Larrea and Freire, 2002).

Ecuador is one of the smallest and less developed South American countries, which is characterized by large social and ethnic disparities (Larrea and Kawachi, 2005; United-Nations-Development-Programme, 2013). In Ecuador, from a national representative survey conducted in 2004, high rates of stunting in the first months of life (3.2% for children of 0-5 months) with a linear increase until two years of life (28.4% for children of 12-23 months) were described. On the other hand, rather low rates of wasting (from 2.1% for children of 0-5 months to 4.6% for children of 12-23 months); while child overweight prevalence varied from 5.3% for children aged 0-5 months to 4.1% for children aged 12-23 months were also reported (World-Bank, 2007). Although higher rates of poverty and malnutrition had been observed amongst rural areas and indigenous households (Hall and Patrinos, 2006; World-Bank, 2007), the assessment of urban inequities could provide cultural and socio-demographic inputs to define the causality of child malnutrition in the Ecuadorian context.

## 1.2. Child feeding patterns in Ecuador

### 1.2.1. Breastfeeding and complementary feeding patterns

The World Health Organization (WHO) recommends that infants should be exclusively breastfed up to six months of age, and continued breastfed up to 2 years old. At six

months, complementary foods must be introduced to the child's diet in addition to breast milk (WHO/PAHO, 2003). Thus, infancy stages could be classified according to their feeding patterns as follows:

- **Exclusive breastfeeding:** Children receive only breast milk. Oral rehydration salts (ORS), vitamins, minerals and medicines are allowed (WHO, 2008).
- **Predominant breastfeeding:** Children receive breast milk as the predominant source of nourishment. Certain liquids (water and water-based drinks, fruit juice), ritual fluids and ORS, vitamins, minerals and medicines are allowed. Non-human milk and food-based fluids are considered part of predominant breastfeeding (WHO, 2008).
- **Complementary feeding:** Infant receive breast milk and solid or semi-solid foods that could include non-human milk and formula (WHO, 2008).
- **Non-breastfeeding (weaning):** Non-breastfed children who receive solid or semi-solid foods, including non-human milk and formula.

The quality of the complementary foods is usually evaluated in function of the meal frequency; amount of food per meal, energy and nutrient density; dietary diversity and biological utilization (UNICEF, 1990; WHO, 2008). In addition, socio-economic status, resource endowment and seasonal variations will also influence the dietary quality (Bermudez and Tucker, 2003; World-Bank, 2007).

Recommendations and patterns of complementary feeding can considerably differ among countries (Michaelsen *et al.*, 2010). In general, cereals represent the main contributor of dietary energy in Latin American countries, followed by tubers and an important contribution of vegetables and fats (Bermudez and Tucker, 2003). In Ecuador, different dietary patterns between highland and coastal children have been reported and further related to stunting prevalence (Leonard *et al.*, 2000). The quality of complementary foods was negatively associated with linear growth and this was more noticeable amongst children until 12 months of age of the highlands. In both, highlands and coastal region, foods at complementary and weaning feeding stage were mostly cereal and tuber-based. However, at the coastal region nutrient density and diet diversity were higher particularly due to the inclusion of animal foods such as eggs, meat and milk (Leonard *et al.*, 2000). Similar results were presented in the World Bank report based on an Ecuadorian representative survey conducted in 2004 (World-Bank, 2007). According to this study, the traditional diet of highland indigenous communities is characterized by low diversity. Main staple foods included cereals (mainly barley flour), tubers (especially potatoes), and beans (fava beans). Rice and maize are usually consumed at lower altitudes, while animal products are considered luxury goods. Furthermore, in the rural highlands, due to fuel limitations, processed foodstuffs that require less time to cook (e.g. pasta) are preferred and tend to replace locally produced

commodities. Other processed foods such as oat flakes become common probably due to their convenience to prepare thick beverages and soups (World-Bank, 2007).

Breastfeeding in developing countries usually continues until 23 months (Arimond and Ruel, 2004). In Ecuador, large variations in breastfeeding cessation have been observed between provinces, ranging from 8 to 23 months, and those differences were mainly culturally and socially related (World-Bank, 2007). However, more recent data are required to effectively evaluate the influence of urbanization on child care and feeding practices. In Latin America, the positive effects of urbanization on maternal education, working opportunities and household economy have been demonstrated (Ruel, 2000; Larrea and Freire, 2002). Consequently, this could lead to greater access to food and more diverse diets. Nevertheless, child care practices and feeding patterns can be negatively influenced due to maternal time constraints. In particular, the median duration of breastfeeding (exclusive or non-exclusive) is usually shorter in urban than in rural areas (Ruel, 2000). In Ecuador, a 40% compliance to exclusive breastfeeding for infants under 6 months of age was reported, as well as the fact that this prevalence was usually higher amongst indigenous and poor households at the highlands (World-Bank, 2007). It has also been suggested that differences in growth patterns between urban and rural areas may be related to food availability, food access, and food utilization (Ruel, 2000).

### 1.2.2. Food chemical hazards and mycotoxin occurrence in staple foods

The statement of *food security* emphasizes the access to safe food (FAO, 2003). Food safety is a public health concern in both developed and developing countries because of the array of food-borne hazards that pose risks to human health and obstacles to international trade (FAO/WHO, 2006; Akkerman *et al.*, 2010). In developing countries, microbiological hazards i.e. acute food and water-borne diarrheal diseases are the cause of death of about 2.2 million people each year, most of them children (FAO/WHO, 2006). On the other hand, chemical hazards are also of important health concern because those conventionally cause long-term adverse effects and their levels in foods after the point of introduction do not significantly change (FAO/WHO, 2006; Garcia-Cela *et al.*, 2012). Mycotoxins are particular chemical hazards because they have a microbiological origin, thus their levels in foods might increase if appropriate conditions for fungal growth are present (Garcia-Cela *et al.*, 2012).

Mycotoxins are naturally occurring toxins considered to be among the most important food chemical contaminants due to their negative impact on public health, food safety and significant economic losses associated with human health, animal productivity and both domestic and international trade (Kuiper-Goodman, 1999; Pennington, 2004; Zollner and Mayer-Helm, 2006). Mycotoxins could affect many diverse cellular processes and have a wide spectrum of toxicological effects including immunosuppression and carcinogenicity (Kuiper-Goodman, 2004; Garcia-Cela *et al.*,

2012). The mycotoxins of most importance worldwide are the aflatoxins, fumonisins, ochratoxin, deoxynivalenol and zearalenone. Those mycotoxins are usually present in a large fraction of food crops such as nuts, cereals and cereal products, dried fruits, fruit juices, spices and coffee.

The chronic exposure to mycotoxins is more likely in low- and middle-income countries, where pre- and post-harvest surveillance is less available because of economical and technological constraints and where, coincidentally, climate conditions favor fungal growth and mycotoxin production (Murphy *et al.*, 2006; Shephard, 2008b; Sherif *et al.*, 2009; Bhat *et al.*, 2010; Wild and Gong, 2010). On the other hand, effective mycotoxin management that involves the application of modern agricultural practices and the enforcement of regulations for food marketing has greatly reduced mycotoxin exposure amongst populations in the developed world (Shephard, 2008b). Public health concern differs among low-income countries. For instance, mycotoxin regulations have been established in about 100 countries, out of which 15 are African (Van Egmond *et al.*, 2007; Wagacha and Muthomi, 2008). In contrast, in other low-income countries, mycotoxin contamination has not been prioritized or even addressed from a public health perspective. Surprisingly, better surveillance is given towards the compliance of rigorous trade regulations rather than from a concern to protect the population locally producing and consuming the contaminated crops (Shephard, 2008b; Wild and Gong, 2010).

Given its ecological diversity and poor food safety chain management, Ecuador is one of the developing countries that could provide favorable conditions for fungi development and mycotoxin production (Pacin *et al.*, 2002). Very limited published data are available about mycotoxins occurrence in staple foods in Ecuador (FAO, 2004; Scussel, 2004), and particularly no information exists on mycotoxin exposure in children, who constitute the population of major health concern in this study.

## 1.3. Mycotoxin contamination

### 1.3.1. Generalities

The agricultural problems associated with fungal contamination and mycotoxins have been noted since ancient times, but recently it has been recognized as a significant health and economical hazard. Human mycotoxicoses have probably occurred since the development of settled agricultural communities reliant on grain stores. It has been suggested that the biblical tenth plague, i.e. the death of the eldest sons over three millennia ago was a consequence of a food-borne disease caused by the massive consumption of contaminated grains with macrocyclic trichothecene mycotoxins (possibly from *Stachybotrys atra*) (Shephard, 2008b). In the tenth century, another ancient mycotoxin outbreak was the known St. Anthony's fire that caused thousands of deaths in many parts of Europe due to consumption of rye contaminated with the ergot

alkaloids produced by the mould *Claviceps purpurea*. During the Second World War the hemorrhagic syndrome known as alimentary toxic aleukia caused thousands of deaths due to consumption of contaminated cereal by T-2 toxin produced by *Fusarium sporotrichioides* and *F. poae*. Although these evidences, it was not before 1960 that mycotoxins were identified as potential health hazard. In this year, the aflatoxins-related X-disease killed 100,000 turkeys after consuming groundnuts infected by *Aspergillus flavus* and *Aspergillus parasiticus* (FAO/IAEA, 2001; Zollner and Mayer-Helm, 2006; Shephard, 2008b).

In general, mycotoxins are compounds of low molecular weight (mostly below 700 Da). Their chemical structure is very diverse, being derivatives of pyrones, anthracinones, coumarines, macrolides, steroids and cyclic polypeptides, amongst others (Sherif *et al.*, 2009). Mycotoxins are produced by about 200 identified filamentous fungal species, growing under a wide range of climatic conditions on agricultural commodities in the field and during storage. Their biological conversion products are also referred as mycotoxins. More than 350 different types of mycotoxins have been discovered, which differ highly in chemical and physicochemical properties, although most of them offer considerable thermal and chemical stability (FAO/IAEA, 2001; Zollner and Mayer-Helm, 2006; Krska *et al.*, 2008; Songsermsakul and Razzazi-Fazeli, 2008; Yazar and Omurtag, 2008; Brase *et al.*, 2009; Sherif *et al.*, 2009).

Four types of toxigenic fungi have been identified: i) plant pathogens, such as *Fusarium graminearum*; ii) fungi that produce mycotoxins on senescent or stressed plants, such as *Fusarium verticillioides* and *Aspergillus flavus* on maize; iii) fungi that colonize the plant and predispose the commodity to mycotoxin contamination after harvest e.g. *Aspergillus flavus* in subtropical maize, and iv) fungi that are found in the soil or decaying plant material that occur on the developing kernels in the field and later proliferate in storage if conditions are suitable, e.g. *Penicillium verrucosum* on cereals and *Aspergillus flavus* on many commodities (Miller, 2008).

Human exposure is mainly caused by either direct contamination through consumption of mycotoxins with plant-origin food that has been spoiled by toxigenic molds (before harvest or at storage) or by carry-over of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feed (Kuiper-Goodman, 1999; Zollner and Mayer-Helm, 2006; Berthiller *et al.*, 2007).

The consumption of foods and feeds contaminated with high levels of mycotoxins may lead to acute and/or chronic adverse health effects in animals and humans (Bhat *et al.*, 2010). These substances may affect many target organs and systems, particularly the liver, kidneys, nervous system, endocrine system and immune system. Some mycotoxins have been classified by the International Agency for Research on Cancer (IARC) as human carcinogens or possible human carcinogens (Kuiper-Goodman, 1999; FAO/IAEA, 2001; Hussein and Brasel, 2001).

### 1.3.2. Mycotoxins of health concern

Based on their known and suspected effects on human and animal health and their negative implications on agricultural productivity, occurrence on staple food crops, the most important mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, HT-2 and T-2 toxins (FAO/IAEA, 2001; Shephard, 2008a, b; Bhat *et al.*, 2010).

#### 1.3.2.1. Aflatoxins

Aflatoxins are a group of difuranocoumarin derivatives synthesized in warm and humid regions around the world by relatively few fungal species of *Aspergillus*, mainly *A. flavus* and *A. parasiticus*. The optimal growth of both species occurs within a temperature range of 10-43°C and at high water activity (0.82-0.99). Aflatoxins are produced within a temperature range of 15-37°C by *A. flavus* and about 28°C by *A. parasiticus*. Aflatoxins are found in a wide variety of foodstuffs like dried fruits, spices, nuts and cereals, and many other agricultural commodities. From about 20 different types identified, $B_1$, $B_2$, $G_1$, $G_2$ and $M_1$ are the major aflatoxins due to their occurrence and toxicity. *A. flavus* typically produces aflatoxin $B_1$ and $B_2$ before harvest and during storage; while *A. parasiticus* produces aflatoxin $G_1$ and $G_2$ besides the B aflatoxins (Figure 1.2) (FAO/IAEA, 2001; Nicholson, 2004; Horn, 2005; Songsermsakul and Razzazi-Fazeli, 2008; Bhat *et al.*, 2010).

Aflatoxins are considered to have the most severe impact of all mycotoxins on human health (Songsermsakul and Razzazi-Fazeli, 2008; Bhat *et al.*, 2010). Aflatoxins are procarcinogenic, mutagenic, teratogenic, immunosuppressive and hepatotoxic agents. The B group contains a cyclopentanone ring, while G group has a lactone ring. Aflatoxin $B_1$ ($AFB_1$) and aflatoxin $G_1$ ($AFG_1$) have an 8, 9 double bond in the form of a vinyl ether at the terminal furan ring, but not $AFB_2$ and $AFG_2$, making the latter less carcinogenic and considerably less toxic. (FAO/IAEA, 2001; Murphy *et al.*, 2006; Zollner and Mayer-Helm, 2006; Songsermsakul and Razzazi-Fazeli, 2008; Brase *et al.*, 2009).

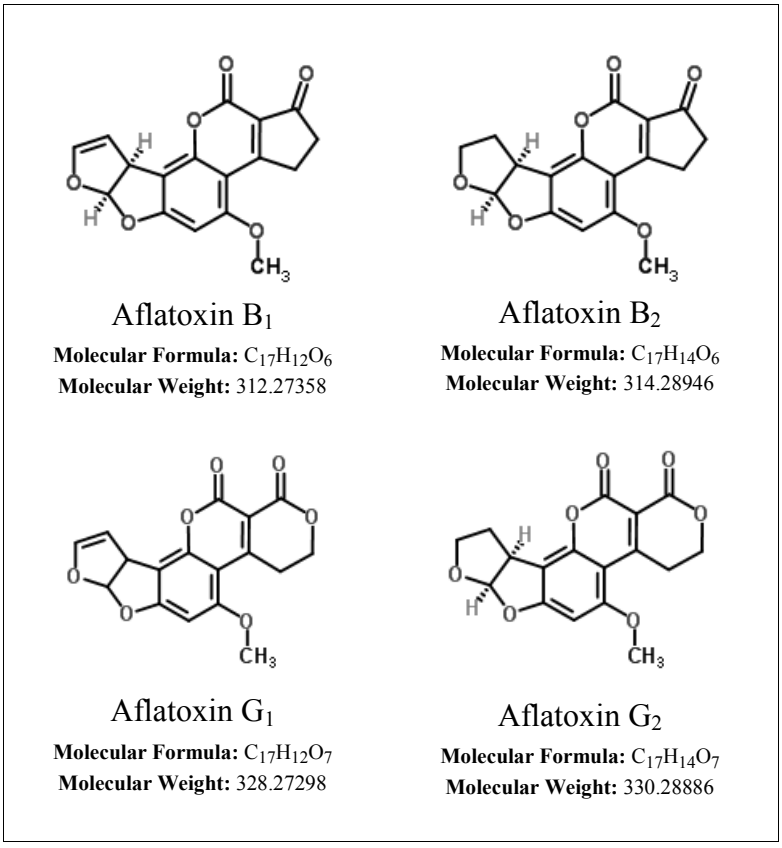



**Figure 1.2.** Chemical structures of aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$ (Source: Royal Society of Chemistry, 2004) (RSC, 2004).

Aflatoxin $B_1$ is metabolized by the P450 system in the liver into epoxide derivates (exo and endo) or into less mutagenic hydroxylated forms such as aflatoxin $M_1$ ($AFM_1$), aflatoxin $P_1$ ($AFP_1$), or aflatoxin $Q_1$ ($AFQ_1$) (Figure 1.3) (FAO/IAEA, 2001; Murphy *et al.*, 2006; Zollner and Mayer-Helm, 2006; Songsermsakul and Razzazi-Fazeli, 2008; Brase *et al.*, 2009). The hydroxylated metabolites can undergo additional Phase II which involves conjugation with glucoronide and/or sulphate groups. Both, endo- and exo-epoxides are the dominant toxic metabolites and can react with macromolecules potentially causing their dysfunction. $AFB_1$-8,9-exo-epoxide can also bind guanine in DNA forming $AFB_1$-N7-guanine adduct, which can be excreted in the urine and is considered as a reliable biomarker for exposure to $AFB_1$. Another pathway of $AFB_1$-8,9-exo-epoxide is the rapid non-enzymatic hydrolysis to $AFB_1$-8,9-dihydrodiol, which at physiological pH, yield to dialdehydic phenolate ion that forms Schiff bases with primary amine groups in proteins (Bammler *et al.*, 2000; Murphy *et al.*, 2006; Turner, 2013).

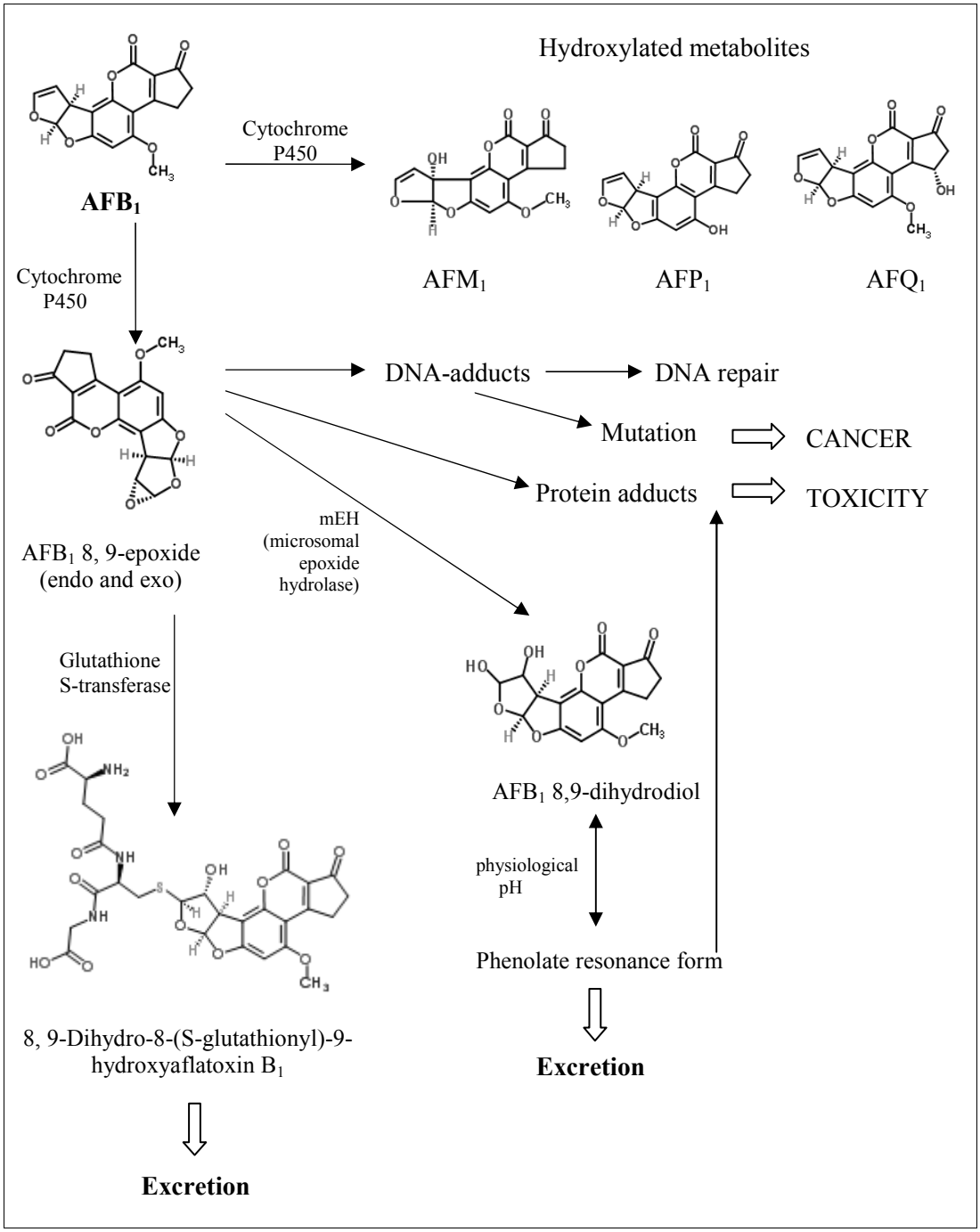



**Figure 1.3.** Overview of the bio-transformations pathways for aflatoxin $B_1$ (Source: Bammler *et al*., 2000) (Bammler *et al.*, 2000).

The carry-over of the hydroxylated derivate $AFM_1$ into the milk of lactating mammals is particularly important for human exposure that can initiate since prenatal life (Galvano *et al.*, 2008; Songsermsakul and Razzazi-Fazeli, 2008; Bhat *et al.*, 2010). $AFM_1$ is a non-inactive detoxification product of $AFB_1$ that is rapidly excreted (within 12-48 hours after ingestion). Adverse effects of $AFM_1$ in breastfed children might include immunologic and nutritional consequences, however toxicological data on

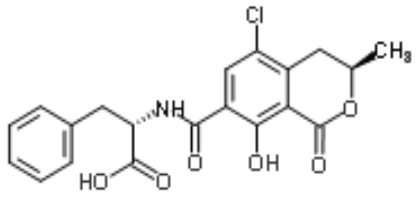
humans are still insufficient (Verma, 2004; Williams *et al.*, 2004; Galvano *et al.*, 2008). Based on animal studies, the potency of $AFM_1$ is considered to be, as conservative estimate, 10% of the potency of aflatoxin $B_1$ (FAO/WHO, 2001).

Among all mycotoxins, aflatoxins are the most toxic and the strongest natural carcinogens (FAO/IAEA, 2001; Shephard, 2008b). In the latest evaluation of the IARC (2002), the naturally occurring mixtures of aflatoxins (as a group) were classified as Group 1: human carcinogens (IARC/WHO, 2002).

### 1.3.2.2. Ochratoxins

Ochratoxins are pentaketides consisting of a dihydroisocoumarin coupled with L-phenylalanine. Ochratoxins are considered the major group of mycotoxins after the discovery of aflatoxins. Ochratoxin A (OTA) is the second most important mycotoxin due its toxicity and common occurrence (Figure 1.4). OTA is mainly produced during storage by *Penicillium verrucosum* and *Aspergillus ochraceus* at temperate and warmer climates, respectively (Zollner and Mayer-Helm, 2006; Brase *et al.*, 2009; Bhat *et al.*, 2010). *A. ochraceus* grows at a relatively low water activity (0.79) and within the temperature range of 8-37°C. *P. verrucosum* grows at a minimum water activity of 0.80 and within a temperature range of 0-31°C. Ochratoxin A is produced by *A. ochraceus* within a temperature range of 15-37°C and within 4-31°C by *P. verrucosum* (FAO/IAEA, 2001).

OTA is a natural contaminant of several agricultural commodities, such as cereals and cereal products, dried fruits, spices, grapes, cocoa and coffee. A carry-over of OTA from contaminated feedstuff into the blood and organs of slaughtered animals can also occur (Zollner and Mayer-Helm, 2006; Songsermsakul and Razzazi-Fazeli, 2008).




Ochratoxin A

**Molecular Formula:** $C_{20}H_{18}ClNO_6$
**Molecular Weight:** 403.81302

**Figure 1.4.** Chemical structure of ochratoxin A (Source: Royal Society of Chemistry, 2004) (RSC, 2004).

OTA exhibits genotoxic, teratogenic, immunosuppressive, carcinogenic and particularly potent nephrotoxic effects in experimental animals. In humans, OTA has been associated with the occurrence of Balkan endemic nephropathy. In 1993, the IARC classified OTA as possible human carcinogen (Group 2B) (IARC/WHO, 1993; FAO/IAEA, 2001; Zollner and Mayer-Helm, 2006; Songsermsakul and Razzazi-Fazeli, 2008; Brase *et al.*, 2009). Currently, OTA or its α-metabolite are the most suitable biomarkers of exposure and are measured in plasma or urine (Coronel *et al.*, 2010).

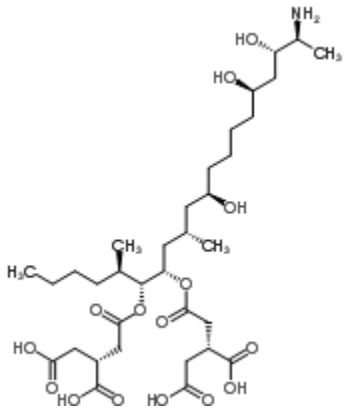
#### 1.3.2.3. *Fusarium* toxins

*Fusarium* species are probably the most prevalent toxin-producing fungi in temperate regions and commonly found on cereals crops such as corn, wheat, barley, rye, rice and oats. *Fusarium* mycotoxins are mainly produced in the field and some toxin synthesis may occur during storage. The most important are Fumonisins, Trichothecenes and Zearalenone (Yazar and Omurtag, 2008).

##### 1.3.2.3.1. Fumonisins

Fumonisins are polyketide derivatives consisting of primary amines with two tricarballylic groups, which contribute to their high water solubility (Songsermsakul and Razzazi-Fazeli, 2008; Brase *et al.*, 2009). Unlike other mycotoxins, fumonisins do not have an aromatic structure or a unique chromophore for easy analytical detection (Murphy *et al.*, 2006). Fumonisins are mainly produced by fungi species as *Fusarium verticillioides* (formerly *F. moniliforme=Gibberella fujikuroi*), *F. proliferatum* and at very low levels by *Alternaria* (Songsermsakul and Razzazi-Fazeli, 2008; Yazar and Omurtag, 2008). *F. moniliforme* grows at a relatively high water activity (0.87-0.99) and within a temperature range of 2.5-37°C, being optimal between 22.5-27.5°C (FAO/IAEA, 2001).

Twenty-eight fumonisins (divided in four series: A, B, C and P) have been isolated, from which Fumonisin $B_1$ ($FB_1$) is the most prominent mycotoxin of this group due to its toxicity and abundance. $FB_1$ is a diester of propane-1, 2, 3-tricarboxylic acid and a pentahydroxyeicosane (Figure 1.5). It is relatively heat stable and occurs as a natural contaminant of cereals, particularly in maize and maize products all over the world under warm and dry conditions (Songsermsakul and Razzazi-Fazeli, 2008; Yazar and Omurtag, 2008; Bhat *et al.*, 2010).



Fumonisin $B_1$

**Molecular Formula:** $C_{34}H_{59}NO_{15}$
**Molecular Weight:** 721.82996

**Figure 1.5.** Chemical structure of fumonisin B1 (Source: Royal Society of Chemistry, 2004) (RSC, 2004).

Different toxicity and carcinogenic mechanisms for $FB_1$ have been suggested. $FB_1$ generates lipid oxidation and peroxidation processes which indirectly could cause DNA damage, as observed in experiments with rats. Also, altered concentrations of cytokines (*in vitro*) and animal antibodies (*in vivo*) suggest a role of fumonisins in immunomodulation. Another mechanism involves the disruption of sphingolipid biosynthesis, which affects several cell functions and signaling pathways such as apoptosis and mitosis. The altered sphingolipid metabolism causes changes in the sphinganine (Sa) to sphingosine (So) ratio (Sa:So), increasing tissue concentrations of Sa. Sphingosine/sphinganine ratio in serum has been used as biomarker of fumonisin exposure; however recently studies have shown that there are no clear relations with different degrees of fumonisin exposure. Moreover, it must be considered that the majority of the fumonisin is excreted unmetabolized in the urine and feces due to their low absorption and short half-life (Murphy *et al.*, 2006; Wild and Gong, 2010).

Fumonisins are non-genotoxic carcinogens that cause severe disorders in animals, as equine leukoencephalomalacia, pulmonary edema in swine and esophageal and hepatic cancer in horses and rats. Fumonisins, which inhibit the uptake of folic acid via the folate receptor, have also been implicated in the high incidence of neural tube defects in rural populations known to consume contaminated maize. Also it has been linked to the occurrence of human esophageal cancers in South Africa, Central America and China; however there is still unclear evidence on their long-term toxicity. $FB_1$ was classified by the IARC as possible carcinogenic in humans (Group 2B) (FAO/IAEA, 2001; IARC/WHO, 2002; Zollner and Mayer-Helm, 2006; Songsermsakul and Razzazi-Fazeli, 2008; Brase *et al.*, 2009; Bhat *et al.*, 2010).

### 1.3.2.3.2. Trichothecenes

Trichothecenes are toxic sesquiterpenoids composed of a central core of fused cyclohexene/tetrahydropyran rings and their toxicity is attributed to a $C_{12}$ -$C_{13}$ epoxide and to a $C_9$-$C_{10}$ double bond (Yazar and Omurtag, 2008; Foroud and Eudes, 2009). Around 190 different structures of trichothecenes have been identified and they are divided in four groups: type A (without a carbonyl group at $C_8$; e.g. T-2 toxin and HT-2 toxin); type B (with a carbonyl group at $C_8$; e.g. deoxynivalenol and nivalenol), type C (with a second epoxy group; e.g. crotocin and baccharin) and type D (with a macrocyclic structure; e.g. roridin A and verrucarin A) (Zollner and Mayer-Helm, 2006; Yazar and Omurtag, 2008; Foroud and Eudes, 2009). Contamination with Type A and B trichothecenes is mainly occurring in cereals (maize, oats, barley and wheat) that are infected by *Fusarium* fungi. The occurrence of type C and D in food and feed is very rare (Zollner and Mayer-Helm, 2006). The toxicity of trichothecenes is based on mechanisms of inhibition of protein synthesis. Trichothecenes exposure can lead to a wide range of effects in humans and animals, such as reproductive dysfunction in mammals and inhibition of seedling growth/regeneration in plants due to anomalies of protein synthesis; immunosuppression linked to an inhibitory effect on the biosynthesis of macromolecules, and the induction of apoptosis in animal cells (Zollner and Mayer-Helm, 2006; Foroud and Eudes, 2009). Due to the insufficient evidence, trichothecenes were classified by the IARC as non-human carcinogens (Group 3) (IARC/WHO, 1993). Based on their high toxicity and occurrence, deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins are considered the most representative trichothecenes (Figure 1.6) (Zollner and Mayer-Helm, 2006; Foroud and Eudes, 2009).

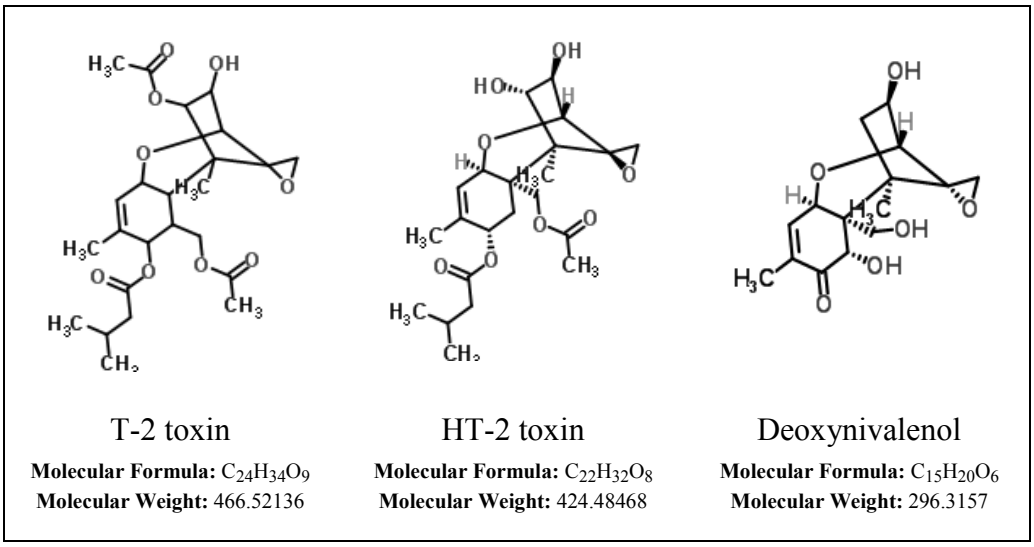



**Figure 1.6.** Chemical structures of the main trichothecenes (Source: Royal Society of Chemistry, 2004) (RSC, 2004).

***Deoxynivalenol***

Deoxynivalenol (DON) is the most prevalent trichothecene in food and feed usually at the highest concentrations compared to other mycotoxins. DON is produced by *F. graminearum*, which growth optimally within 24-26°C and at high water activities (0.9-0.99) (FAO/IAEA, 2001; Pestka, 2010). DON is colloquially known as vomitoxin because of emetic effects in pigs as a result of acute poisoning. In experimental animal models (pigs, rats and mice), chronic DON's toxicity mechanisms have been associated with anorexia, growth retardation, immunotoxicity, impaired reproduction, teratogenesis and cytotoxicity including disruption of macromolecular synthesis, cell signaling, differentiation, proliferation, gene up-regulation and apoptosis. Insufficient data about the human exposure and responses to this toxin are available (Murphy *et al.*, 2006; Pestka, 2010). However, the effects of DON are most pronounced in acute than chronic exposures. The intake of single meal containing high concentrations of DON could cause gastroenteritis with vomiting in humans with a dose around 50 μg $kg^{-1}$ bw. No epidemiological data are available about chronic effects of DON in humans.

Recent studies have suggested the use of urinary biomarkers of DON (and acetylated derivatives) to assess the actual human exposure to DON (Pestka, 2010; Ng, 2011).

***T-2 and HT-2 toxins***

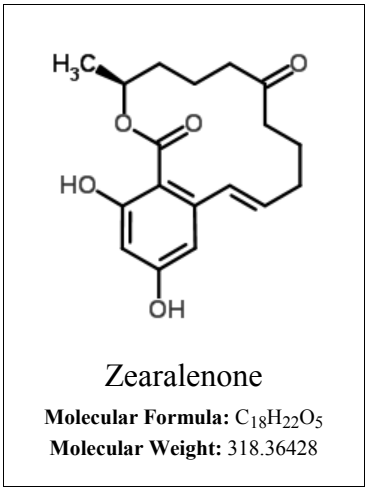
T-2 and HT-2 toxins are type-A trichothecenes and are usually produced in cold climate regions or during wet storage conditions in a variety of cereal grains and cereal products. T-2 and HT-2 toxins are mainly produced by *F. sporotrichiodies* and *F. poae*. The optimal growth of occurs within a temperature range of 22.5-27.5°C and at high water activities (0.88-0.99) (FAO/IAEA, 2001; Visconti *et al.*, 2005).

T-2 toxin is considered the most toxic trichothecene. Its toxicity in mammals is roughly ten times higher than the toxicity of DON (Foroud and Eudes, 2009). T-2 is a potent inhibitor of protein synthesis and mitochondrial function both *in vivo* and *in vitro*, and it has been also related to immunosuppressive and cytotoxic effects (Visconti *et al.*, 2005). The liver is the major organ for the metabolism of T-2 toxin. In particular, hepatic carboxylesterases are responsible for the specific deacetylation of T-2, giving HT-2 as the major metabolite. Limited information is available on the direct toxicity and adverse affects of HT-2 alone (Visconti *et al.*, 2005; Lattanzio *et al.*, 2009).

The consumption of grains contaminated with T-2 toxin is probable the cause of the disease known as alimentary toxic aleukia (ATA) characterized by an initial stage of intestinal irritation causing emesis and diarrhea followed by aleukia and anemia. T-2 is also responsible for outbreaks of hemorrhagic disease in animals and oral lesions and neurotoxic effects in poultry. (IARC/WHO, 1993; FAO/IAEA, 2001; Foroud and Eudes, 2009).

#### 1.3.2.3.3. Zearalenone

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin with a phenolic resorcyclic acid lactone structure (Zollner and Mayer-Helm, 2006) (Figure 1.7). ZEN belongs to the group of fusariotoxins and it is mainly produced by *Fusarium graminearum* and *Fusarium culmorum*, which colonize grains under prolonged cool and wet conditions in temperate and warm regions. It is a stable compound during storage, milling, processing, and cooking (Songsermsakul and Razzazi-Fazeli, 2008) (Zollner and Mayer-Helm, 2006). ZEN has a worldwide distribution and can contaminate mostly cereals like barley, maize, oat, sorghum, wheat, rice and soy beans. The carry-over of ZEN metabolites (α-Zearalenol and α-Zearalanol) into animal tissues, milk and eggs after intake of contaminated food is particular important since considerably higher estrogenic effects than ZEN have been conferred to those metabolites (Zollner and Mayer-Helm, 2006; Songsermsakul and Razzazi-Fazeli, 2008; Bhat *et al.*, 2010). ZEN has the potential to disrupt sex steroid hormone functions and it has been associated with reproductive problems such as vulvar and mammary swelling and infertility in specific animals and possibly in humans. Its hyperestrogenicity is attributed to an agonistic effect on the estrogen receptor (FAO/IAEA, 2001; Murphy *et al.*, 2006; Zollner and Mayer-Helm, 2006; Yazar and Omurtag, 2008). Similar to the trichothecenes, there is limited evidence of carcinogenicity of ZEN in experimental animals, and therefore the IARC classified it as a non-human carcinogen (Group 3) (IARC/WHO, 1993; FAO/IAEA, 2001; Zollner and Mayer-Helm, 2006; Foroud and Eudes, 2009).




Zearalenone

**Molecular Formula:** $C_{18}H_{22}O_5$

**Molecular Weight:** 318.36428

**Figure 1.7.** Chemical structure of zearalenone (Source: Royal Society of Chemistry, 2004) (RSC, 2004).

### 1.3.3. Mycotoxin co-occurrence

The complex ecology for fungal growth and their toxigenic capabilities can lead to the production of several mycotoxins in the same food commodity. In particular, cereals are

highly prone to fungal attack and subsequent contamination of multiple mycotoxins in the field or during storage. Nowadays, the mere presence of several mycotoxins is considered a danger because of the possible additive, synergistic or antagonistic interactions and their subsequent toxicological impact in human bodies (FAO/IAEA, 2001; Brase *et al.*, 2009; Capriotti *et al.*, 2012). Although *in vivo* data on combined toxic effects of mycotoxins are very limited, it has been suggested that mycotoxins with similar mode of action would be expected to have additive effects (Speijers and Speijers, 2004). Some *in vitro* studies have shown that mixtures of mycotoxins can produce cytotoxicity by enhancement (DON, NIV, ZEN & $FB_1$) or synergism (DON & NIV; OTA & $FB_1$; ZEN, $FB_1$ &DON) (Creppy *et al.*, 2004; Kouadio *et al.*, 2007; Wan *et al.*, 2013).

### 1.3.4. Masked mycotoxins

Masked mycotoxins are mycotoxin conjugates that may also occur in foodstuff and result from the metabolism of living plants, particularly from detoxification processes against xenobiotics. Those conjugates have been designated as "masked" because are undetectable by the analytical methods developed for the precursor mycotoxins. Masked mycotoxins can also be formed after food processing and those are usually less toxic than their precursors (Berthiller *et al.*, 2009; Berthiller *et al.*, 2013).

Masked mycotoxins can be either soluble (extractable) or bound (non-extractable) conjugates. Bound mycotoxins are covalently or non-covalently attached to polymeric carbohydrate or protein matrices. The chemical modifications involved in the formation of masked mycotoxins in the plant tissue occur in three phases. Phase I includes the reduction, oxidation or acetylation of the precursor mycotoxin. The resulting products of this phase are reactive molecules that usually exhibit higher toxicity than the precursor. Phase II comprises enzymatic transformation by conjugation, glycosidation and sulfatation of the reactive products (from phase I) into more hydrophilic molecules. Phase II contributes to the detoxification of the living organism by facilitating the elimination of these conjugated products. Phase III comprises the compartmentalization of the mycotoxin conjugates inside the vegetal vacuoles or binding to the cell wall. The metabolic processes of Phase III represent the major difference in the detoxification mechanism between plants and mammals, i.e. mycotoxin conjugates remain present in the plant tissue, while in animals, phase III comprises excretion processes (Berthiller *et al.*, 2009; Vendl *et al.*, 2009; Berthiller *et al.*, 2013; De Boevre *et al.*, 2013).

Conjugates of *Fusarium* mycotoxins (deoxynivalenol, nivalenol, fumonisins, fusarenon-X, T-2 toxin, HT-2 toxin, fusaric acid and zearalenone), ochratoxin A and destruxins have been isolated and structurally identified in plant cell cultures. However, the natural occurrence of few masked mycotoxins has been described to date. In this regard, zearalenone-14-β-D-glucopyranoside (Z14G), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl- deoxynivalenol (15ADON) and

deoxynivalenol-3-β-D-glucopyranoside (D3G) are the main conjugates described in cereals such as wheat, maize and oat (De Boevre *et al.*, 2012; Berthiller *et al.*, 2013). DON conjugates (D3G, 3ADON and 15ADON) usually occur together with their precursor mycotoxin in cereal products. In addition, bound fumonisins were discovered in maize after applying alkaline hydrolysis. Bound fumonisins (also called "hidden") result from the covalent binding between the tricarboxylic moiety and hydroxyl groups of carbohydrates, amino groups of amino acids upon heating. The formation of fumonisin fatty acid esters by the plant could occur. In addition, it has been suggested that hidden fumonisins could also result from physical entrapment into the macromolecular structures of biopolymers such as starch (Dall'Asta *et al.*, 2009; De Boevre *et al.*, 2012; Berthiller *et al.*, 2013).

Masked mycotoxin may exhibit decreased toxicity (e.g. D3G), similar toxicity (e.g. (4R)- and (4S)-4-hydroxy-ochratoxin A) or increased toxicity (e.g. α-ZEL) in comparison with the precursor mycotoxin. Moreover, some masked mycotoxins could turn back into their toxic precursors during gastrointestinal digestion (e.g. ZEN and DON conjugates by the gut microflora in humans). Therefore, the total mycotoxin content in the foodstuff could be underestimated when only the precursor mycotoxin is detected by conventional analytical methods (Berthiller *et al.*, 2009; Berthiller *et al.*, 2013; Dall'Erta *et al.*, 2013; De Boevre *et al.*, 2013).

Currently, legal limits and control strategies only focus on the precursor mycotoxins. Since the presence of masked mycotoxins is an emerging issue, toxicological and occurrence data are still lacking. For the case of DON, the contribution of its conjugates to the total dietary exposure was evaluated by JECFA in 2010. However, additional studies on D3G toxicological pathways, its occurrence and effects of processing on the levels of D3G and ADONs were recommended at that time. So far, some analytical methods have been developed. However, the analysis of masked mycotoxins is still challenging due to several limitations such as lacking of analytical standards, discrimination between the conjugates and the precursor mycotoxins, and chemical or enzymatic treatment to efficiently release bound mycotoxins (Berthiller *et al.*, 2013).

### 1.3.5. Mycotoxin regulations

Several regulations for maximum levels of mycotoxins in foodstuffs intended for human consumption have been harmonized between countries belonging to some economic/trading communities (Table 1.1) (FAO, 2004; Van Egmond *et al.*, 2007). The US Food and Drug Administration (FDA) has enforced regulatory limits in foods and feeds for fumonisins, aflatoxins, patuline and DON (FDA, 2010) (National-Grain-and-Feed-Association, 2011). For the European community, harmonized regulations are available for more than 40 mycotoxin-food combinations (Van Egmond *et al.*, 2007). In Latin America some countries either have national regulations or have adopted proposals of organizations or research institutes from other countries. In this context,

Argentina, Brazil, Paraguay and Uruguay are the South American countries belonging to MERCOSUR (Mercado Común del Sur) that has set common limits for aflatoxins in peanuts, maize and products thereof (FAO, 2004). In 1995 it was reported that Ecuador used the regulations from the Switzerland's Federal Research Station on Animal Products Grangeneve de Poiseaux (Resnik *et al.*, 1995). Nonetheless, in Ecuador no regulations are enforced as stated in the last FAO worldwide report about regulations for mycotoxins in food and feed (2003) (FAO, 2004).

The information of permissible levels of mycotoxins in foods intended for infant consumption is limited. European maximum levels for $AFB_1$ and $FB_1$ in baby foods and processed cereal-based foods for infants and young children are 0.10 μg $kg^{-1}$ and 200 μg $kg^{-1}$, respectively (European-Commission, 2006b). In addition, the European maximum level for the sum of T-2 and HT-2 toxins in cereal-based foods for infants and young children is 15 μg $kg^{-1}$ (European-Commission, 2013). MERCOSUR has not established any regulation for mycotoxins in foods intended for consumption in this population. Amongst South American countries, only Uruguay has established maximum limits for the sum of aflatoxins (3 μg $kg^{-1}$) (FAO, 2004). Regarding $AFM_1$ in milk, European regulations and MERCOSUR have set a maximum limit of 0.05 μg $kg^{-1}$, while a maximum limit of 0.025 μg $kg^{-1}$ in infant milk (formula) is also considered in European regulations.

**Table 1.1.** Maximum permitted levels of the main mycotoxins of health concern in cereals established by the European Commission (European-Commission, 2006b, 2013), FDA (National-Grain-and-Feed-Association, 2011) and in Latin American (LA) countries (FAO, 2004), all expressed in μg $kg^{-1}$.

| Mycotoxin | European Commission | FDA | LA countries |
|---|---|---|---|
| Sum of Aflatoxins ($G_2$+$G_1$+$B_2$+$B_1$) | 4 (all cereals); 10 (rice) | 20 | 10 [a]<br>5 [b] |
| Aflatoxin ($AFB_1$) | 2 (all cereals); 5 (rice) | - | - |
| Ochratoxin A (OTA) | 3 | - | 50 [c] |
| Fumonisin $B_1$ ($FB_1$) | 400 [d] | 2,000 [e] | - |
| Deoxynivalenol (DON) | 750 | 1,000 [f] | 1,000 [g] |
| Sum HT-2 + T-2 toxins | 50 (cereals) [h]; 25 (pasta);<br>200 (oat flakes) | - | - |
| Zearalenone (ZEN) | 75 | - | 200 [b, i] |

[a] Colombian limit for foods in general
[b] Chilean limit for foods in general
[c] Uruguayan limit of OTA for rice

[d] Sum of FB1+FB2, only established for maize based foods
[e] Sum of FB1+FB2+FB3 corresponding to the lowest advisory level for maize
[f] Advisory level of DON for finished wheat products
[g] Uruguayan limit of DON for wheat flour and by-products
[h] Cereal grains for direct human consumption (different than oat and maize) that have undergone drying, cleaning, de-husking and sorting processes and on which no further cleaning and sorting processes will be performed before their further processing in the food chain
[i] Uruguayan limit of ZEN for maize and barley

## 1.4. Risk assessment

### 1.4.1. Infant susceptibility to mycotoxicoses

Exposure to mycotoxins is a serious risk to human health, especially in developing countries where population commonly faces the effects of poverty and deficient quality control of food contamination. Furthermore, in those countries the high exposure to mycotoxins and mycotoxicoses has been associated with malnutrition, leading to the reduction of the immune response and decreasing the biochemical mechanisms for detoxification (FAO/IAEA, 2001; Shephard, 2008b).

The susceptibility to the detrimental health outcomes of mycotoxin exposure can vary in function of the combination of several factors such as body composition, exposure time, age and nutritional status, geographic, cultural and economical conditions, among others (FAO/WHO, 1997; Sherif *et al.*, 2009). Infants and young children are considered to be more susceptible to toxins than adults due to their higher intake/body weight ratio (low body weight), higher metabolic rate and lower detoxification capacity. Besides the incomplete development of some organs and tissues, the higher vulnerability of infants and young children to mycotoxicoses is also related to their limited diet diversity (WHO, 2006a; Sherif *et al.*, 2009). Malnutrition may enhance the dietary exposure to mycotoxins, but also the inverse process has been suggested (Hussein and Brasel, 2001; Kimanya *et al.*, 2010). During the last decades and particularly in developing countries, epidemiological evidence about the relation between aflatoxin exposure and childhood growth faltering, kwashiorkor pathogenesis and immunosuppression has emerged (Williams *et al.*, 2004; Shephard, 2008b; Sherif *et al.*, 2009). Growth faltering, hyperestrogenism, premature thelarche, pubarche, breast enlargement and precocious puberty are adverse effects of the exposure to ZEN during childhood. Recently, the exposure to fumonisins has also been associated to child growth retardation (Kimanya *et al.*, 2010). In addition, the acute exposure to DON and other trichothecenes has been associated to several outbreaks of vomiting illness (Sherif *et al.*, 2009). Furthermore, role of multi-mycotoxin exposure (aflatoxins, fumonisins and deoxynivalenol) in the pathogenesis of environmental enteropathy and impaired nutrient intake has been suggested as an important convergent pathway towards child growth retardation (Smith *et al.*, 2012).

#### 1.4.1.1. Physiological differences between infants and adults

At birth, most body systems such as immune, gastrointestinal, renal, endocrine, and reproductive are structurally or functionally immature. The development of physiological functions, i.e. absorption, distribution, metabolism and excretion, is critical when determining child response to chemicals (Scheuplein *et al.*, 2002; WHO, 2006a).

Absorption of chemicals differs between children and adults. Gastric pH is higher in newborns (pH 6-8) than in adults (pH 1-3), resulting in different ionization and absorption of certain chemicals. The alkaline gastric pH in newborns and infants may lead to enhanced bioavailability of weakly basic compounds but reduced bioavailability of weakly acidic compounds. Gastric acid production at about two years of age becomes similar to the adult production. Water and lipid content of the body differ as a function of age and therefore also the physiological distribution volume for chemicals is different between infants and adults. For instance, infants present a greater dilution of water-soluble chemicals due to their larger extracellular fluid volume, while lipid-soluble chemicals would be distributed in a smaller volume of fat in comparison to adults. Plasma protein concentrations also determined the volumetric distribution of chemicals. Although the total plasma protein concentrations remain constant at different life stages, the concentrations of the specific binding proteins do vary with age being lower in newborn infants (WHO, 2006a). Metabolic rates are generally lower in neonates and young children than in adults. This is mainly related to the gradual synthesis of the cytochrome P450 isoforms which are responsible of the catalysis of the phase I reactions in the liver (Murphy *et al.*, 2006; WHO, 2006a). Limited data are available regarding the ontogeny of phase II enzymes in human tissues. In general, most of the metabolizing enzyme systems seem to be developed from the middle of gestation until a few months after birth. Elimination rates are also generally lower in neonates than in adults, as observed with some indicators of liver function (e.g. bilirubin). Renal clearance is also lower in neonates than in older children and adults, for all chemical classes: lipophilic, hydrophilic, and organic ions. Glomerular filtration rate at normal-term birth is about one third of the adult value when expressed on the basis of body surface area. This function matures in the first six months of life. In contrast, the tubular reabsorption process reaches adult levels within a few days after birth (WHO, 2006a).

### 1.4.2. Dietary exposure assessment

The dietary exposure assessment is part of the integral risk-based approach for the management of public health hazards in food, known as “Risk analysis”. This systematic approach was developed in 1991 by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) (WHO, 1995b). This is a structured decision-making process that comprises three interrelated components: risk management, risk assessment and risk communication (Figure 1.8)

(WHO, 1995a; FAO/WHO, 2006).


**Figure 1.8.** A Relationship between the three components of risk analysis (Source: WHO, 1995. About risk analysis in food. Available at: http://www.who.int/foodsafety/micro/riskanalysis/en/. Accessed: Oct 31st, 2013 (WHO, 1995a).

Regardless of the institutional context, risk analysis is used to estimate the risks for human health and safety, to identify and implement appropriate measures and policies to control the risks, and to communicate with stakeholders about the risks and measures applied, contributing to better food safety outcomes and improvements in public health (FAO/WHO, 2006).

Risk assessment is the scientific-based compound of a risk analysis, and it is a process consisting of: i) hazard identification; ii) hazard characterization, iii) exposure assessment and, iv) risk characterization (FAO/WHO, 2006).

#### 1.4.2.1. Hazard identification

Hazard identification implies the identification of biological, chemical and physical agents capable of causing adverse health effects and may be present in a particular food or group of foods (FAO/WHO, 2006). For the full understanding of the toxic properties of a hazard, it is necessary to conduct toxicological studies (short-term, sub-chronic, chronic, carcinogenic, genotoxic, reproductive) at various dosage levels in a variety of animal species under controlled conditions (Kuiper-Goodman, 2004). Additionally, studies need to be conducted to determine the absorption, distribution, metabolism, and excretion of mycotoxins. For each adequately conducted animal study, the highest dose at which no adverse effects are observed (NOAEL) and the lowest dose with observed adverse effect level (LOAEL) are usually determined (Kuiper-Goodman, 2004; FAO/WHO, 2006).

#### 1.4.2.2. Hazard characterization

Hazard characterization implies the qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with the identified hazard (FAO/WHO, 2006). Hazard characterization is considered as a biological and mathematical extrapolation phase of the risk assessment in terms of dose-response towards a predictive characterization of the hazard to humans under a variety of exposure scenarios (Kuiper-Goodman, 2004). The treatment for hazard characterization of non-carcinogenic mycotoxins is different than for those that are carcinogens. When the toxic effect results from a mechanism that has a threshold, a safe intake level can be established, such as the tolerable daily intake (TDI) which is equivalent to the acceptable daily intake (ADI) used for food additives or pesticide residues.

#### 1.4.2.2.1. Non-carcinogenic chemicals

For non-carcinogenic chemicals, it is assumed that for the adverse effects observed in animals or humans there is a threshold dose (such as the NOAEL), below which these effects are not observed. The TDI is the dose that can be safely consumed daily over a lifetime without incurring appreciable adverse health effects. The TDI is commonly derived from dividing the NOAEL (from animal studies) by a default safety/uncertainty factor of 100 when extrapolating to humans. This factor takes into consideration a default factor of 10 for inter-species differences (replaced by two factors: a 4-fold factor and a 2.5-fold factor for toxicokinetic and toxicodynamic differences, respectively), and another 10-fold factor for inter-individual variation in susceptibility amongst humans (replaced by two factors: two 3.2-fold factors to account for toxicokinetic and toxicodynamic differences each). For infants and young children, due to their possible greater vulnerability, an additional 10-fold uncertainty factor has been suggested as a default, unless comprehensive data for this group are available (Kuiper-Goodman, 2004; FAO/WHO, 2006; Garcia-Cela *et al.*, 2012).

#### 1.4.2.2.2. Carcinogenic chemicals

For carcinogenic chemicals, no threshold dose is proposed because there is always some risk of cancer initiation even at very low doses, unless it can be clearly established that the mode of action involves an indirect mechanism that may have a threshold (Kuiper-Goodman, 2004; WHO, 2006a). Furthermore, carcinogenic mycotoxins must be differentiated between two groups, DNA reactive (genotoxic carcinogens) and non-DNA reactive (epigenetic carcinogens) compounds. Tumors induced through a non-DNA-reactive mode of action are typically less aggressive than those produced by DNA-reactive chemicals. The mode of action of the DNA reactive chemicals involves mutations in key genes that control cellular processes such as proliferation. Tumor formation induced by non-DNA reactive chemicals involves epigenetic events such as cytoxicity, hormonal balance, chronic inflammation leading to free radicals and DNA damage. For the latter group, there is a level of exposure below which the cellular events triggering the carcinogenic process are not disturbed, and therefore a NOAEL

can be defined from toxicological studies (Kuiper-Goodman, 2004; Pratt *et al.*, 2009). Even though, it is assumed that cancer induced in animals by both DNA-reactive and non-DNA-reactive chemicals is relevant to humans by default. For DNA-reactive carcinogens there is no threshold dose below which this reaction does not take place. However, it has been suggested that some of those DNA-reactive chemicals may have a threshold since experimental data on cell responses have emerged (Pratt *et al.*, 2009).

Recently, the use of the benchmark dose (BMD) approach for the estimation of margins of exposure for both genotoxic and carcinogenic mycotoxins in risk characterization has been proposed by the Scientific Committee of the European Food Safety Authority (EFSA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (EFSA, 2009) (Pratt *et al.*, 2009). The BMD is the dose, derived from the dose-response curve, which results in a predetermined level of adverse response (critical effect size). The lower confidence limit of the BMD (BMDL) is often taken as the starting point for determining allowable exposure levels (Figure 1.9) (Travis *et al.*, 2005). The use of this alternative approach has been encouraged since it offers several advantages including that the BMD is not limited to experimental doses selected by the investigators. In addition, it is derived from all the experimental data in the observable range of the dose-response curve, and its BMDL properly addresses the uncertainty of a study (Muri *et al.*, 2009). The BMD cannot be considered as a true starting point in the dose-response curve; instead it represents one point in a continuum of response incidences versus dose. In the dose-response curve, the BMD is in the same region as the NOAEL, and therefore it can be treated in a similar way (Kuiper-Goodman, 2004).

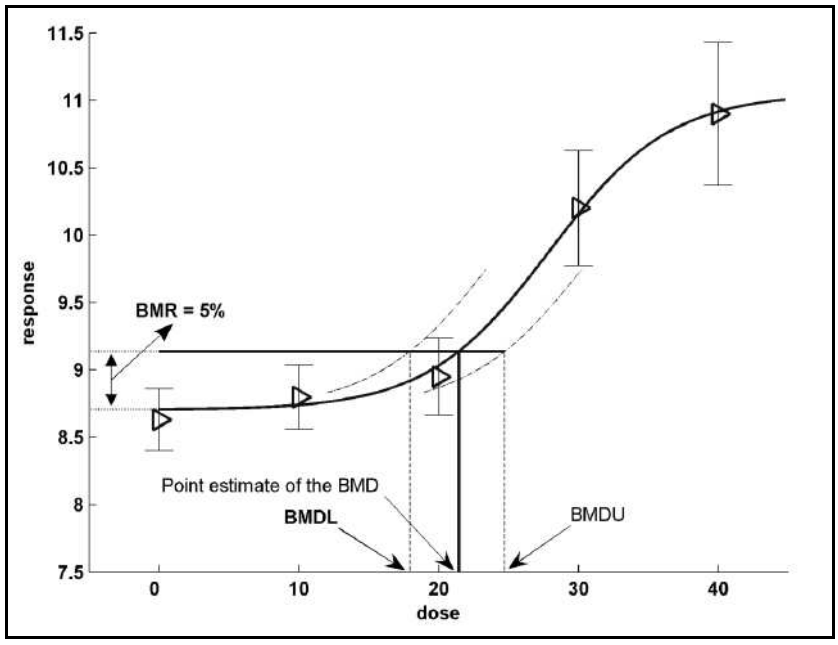



**Figure 1.9.** Illustration of the BMD approach (Source: EFSA, 2009) (EFSA, 2009).

It is noteworthy that although no agency has set a TDI for $AFB_1$, published studies of risk assessment usually compare the exposure to $AFB_1$ with the PMTDI (provisional maximum tolerable daily intake) of 0.001 μg $kg^{-1}$ bw per day that was suggested by Kuiper-Goodman in 1998 (Kuiper-Goodman, 1998; Park *et al.*, 2004; Leblanc *et al.*, 2005; Villa and Markaki, 2009).

An overview of the carcinogenic potential assigned to mycotoxins of health concern and their toxicological thresholds are presented in Table 1.2 (Kuiper-Goodman, 2004; Garcia-Cela *et al.*, 2012).

**Table 1.2.** Carcinogenic potential and toxicological thresholds for selected mycotoxins of health concern.

| Mycotoxin | Carcinogenic potential | Toxicological threshold (μg $kg^{-1}$ bw per day) | Reference |
|---|---|---|---|
| Aflatoxin $B_1$ | NTC | PMTDI = 0.001 | (Kuiper-Goodman, 1998) |
| Ochratoxin A | NTC / TC | PTDI = 0.014 | JECFA, 2007 (JECFA, 2007) |
| Fumonisins [a] | TC | PMTDI = 2 | SCF, 2003 (SCF, 2003) |
| Zearalenone | TC | PTDI = 0.25 | EFSA, 2011 (EFSA, 2011b) |
| Deoxynivalenol | NC | TDI = 1 | SCF, 2002 (SCF, 2002) |
| HT-2 + T-2 | NC | TDI = 0.1 | EFSA, 2011 (EFSA, 2011a) |

NTC, non-threshold carcinogen (DNA reactive)
TC, threshold carcinogen (non-DNA reactive)
NC, non-carcinogenic
[a] $FB_1$, $FB_2$ and $FB_3$, alone or in combination.

### 1.4.2.3. Exposure assessment

Exposure assessment implies a qualitative and/or quantitative evaluation of the likely intake of the identified hazard (FAO/WHO, 2006). In the dietary exposure assessment, food consumption data are combined with the contamination level of the mycotoxins in food. The outcome of the assessment is usually expressed as the total weight of contaminant ingested together with the foodstuff, per kilogram of body weight on a daily basis (FAO/WHO, 1997; Pennington, 2004; FAO/WHO, 2006; Sherif *et al.*, 2009). Besides body composition, the susceptibility to the detrimental health outcomes of a chemical hazard can vary in function of the combination of several factors such as exposure time, age and nutritional status, among others (FAO/WHO, 1997).

The quantity and quality of the data used as input for an exposure assessment must be carefully assessed. The most commonly used and appropriate source of food

consumption data for exposure assessments is food consumption surveys on individuals. Combined data from duplicate 24-hour recall or weighted record with food-frequency questionnaires is preferable because it meets the purpose of both nutrition and exposure assessment without overestimation (Lambe, 2002; Kuiper-Goodman, 2004; Pennington, 2004; FAO/WHO, 2006).

Food surveillance for contaminants may be difficult to match with estimates of food consumption. To perform exposure assessments, reliable analytical data of the occurrence of mycotoxins in food commodities that were appropriately sampled are required. When the purpose of the analysis is assessing compliance with regulations, raw products are sampled. However, those foods do not correspond to what is normally eaten and some reduction (but not complete removal) of the mycotoxin levels in foodstuff is expected due to processing and cooking (Lambe, 2002; Kuiper-Goodman, 2004; Bhat *et al.*, 2010; Duarte *et al.*, 2010; Voss and Snook, 2010). Mycotoxins in foods frequently occur at low concentrations. Therefore, a high percentage of the food samples analyzed could have values below the limit of detection (LOD). The "non-detects" are usually treated in three different ways: i) equal to the LOD (producing a positive bias); ii) equal to zero (producing a negative bias), or iii) equal to half the LOD (may produce a positive or negative bias) and those different scenarios must be evaluated and reported (Lambe, 2002; Kuiper-Goodman, 2004).

Quantitative exposure assessment is characterized by assigning a numerical value to the risk from a model based on the contamination and consumption data. Depending on the type of data available, several methods can be used to estimate the risk such as: i) deterministic modeling (point-estimate); ii) simple distribution method and iii) probabilistic modeling (Lambe, 2002; Baert *et al.*, 2009).

#### 1.4.2.3.1. Deterministic modeling

Deterministic modeling is based on conservative assumptions and uses a single point estimate of each variable within the model to determine the respective outcome (Kuiper-Goodman, 2004; Baert *et al.*, 2009). Deterministic modeling is also called point-estimate approach. To calculate the exposure to a contaminant, a fixed value for food consumption (e.g. mean or maximum consumption) is multiplied by a fixed value for the concentration of the contaminant in that food (e.g. mean level or permitted level according to the legislation). Deterministic modeling is commonly used as the first step in exposure assessment considered as an inexpensive screening tool for identifying contaminant for which safe intake limits may be exceeded. This conservative approach is adopted when food consumption data at individual level are not available. Also, when occurrence data are not available, deterministic modeling is adopted assuming that the chemical is present in all the products at the maximum level permitted by regulations. The deterministic approach has the disadvantage of providing only limited information for risk management because it is assumed that the contaminant is always present in the

food and therefore, this conservative approach tends to overestimate the actual exposure since it is designed to cover the worst-case scenario (Lambe, 2002; Kuiper-Goodman, 2004; Baert *et al.*, 2009).

#### 1.4.2.3.2. Simple distribution method

The simple distribution method uses the food intake distribution (from consumption surveys) and a fixed (single) value that represent the chemical concentration in the food. This approach could be applied in case of scarce concentration data. The outcomes of this approach are more informative than point estimates because the variability of the food consumption distribution is considered. However, this approach still retain conservative assumptions related to the presence and concentration of the chemical, and therefore can only be considered to give an estimation of the exposure in a worst-case scenario. (Lambe, 2002; FAO/WHO, 2009).

#### 1.4.2.3.3. Probabilistic modeling

Nowadays, probabilistic modeling is used to provide more realistic estimates of exposure to food hazards. This approach aims to describe the exposure distribution for a given population by quantifying the range of exposure and the degree of likelihood of each exposure level. Numerical simulations are processes of replication of the reality that considers the propagation of variability and uncertainty of input variables through a model and is based on simulated random sampling (Kuiper-Goodman, 2004).

### 1.4.2.4. Monte Carlo simulation

Monte Carlo simulation is a numerical simulation method used by the JECFA for the safety evaluation of mycotoxins in food. In this method, one random sample from each input distribution is selected (food intake and contamination data) and the set of samples is entered into the deterministic model (point estimate). Then, the model is computed, as it would be for any deterministic analysis and the result is stored. This process, called iteration, is repeated several times until the total number of iterations selected *a priori* is completed. Finally, the results of these iterations are aggregated and the probability distribution is calculated. These distributions give the likelihood and the magnitude of dietary exposure levels (FAO/WHO, 1997; Kuiper-Goodman, 2004; Baert *et al.*, 2009).

The input distributions can be distributional functions (parametric approach) or data as such (non-parametric approach). For the parametric approach, the data are fitted to a distribution function, such as the normal, gamma, binomial distribution, etc. The best-fit input distribution function is selected by applying statistical goodness-of-fit tests and by graphical inspection. In case there are not enough data points to fit a distribution or no acceptable fit can be obtained, the original data are used as an input for the model (non-parametric approach) (FAO/WHO, 1997; Kuiper-Goodman, 2004; Baert *et al.*, 2009).

Probabilistic exposure assessment has the advantage of providing meaningful information for risk managers based on the whole distribution of exposure, from minimum to maximum and at different percentiles. Additionally, all sources of variability and uncertainty are considered by this approach, allowing a better understanding of the exposure distribution and targeting of the population at higher risk. Variability is a non-reducible property that represents the heterogeneity in a well-characterized population. Uncertainty represents the partial ignorance or lack of perfect information about the characterized phenomena or model that can be reduced through further measurements or study (Kuiper-Goodman, 2004; FAO/WHO, 2009). An important disadvantage of the probability risk assessment is the need of accurate prediction of the tails in a distribution, which are very important since the evaluation of toxicity focuses typically at the largest-exposure concentrations (e.g. $95^{th}$ percentile) (Kuiper-Goodman, 2004; Baert *et al.*, 2009; FAO/WHO, 2009).

#### 1.4.2.5. Risk characterization

Risk characterization implies a qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment (FAO/WHO, 2006). For chronic exposure, the duration, the extent of the higher exposure, toxicokinetics and mode of action will determine the degree of the risk. Even if low contamination levels are present, the risk of long-term low-level exposure is important given that mycotoxins affect mainly staple foods (Kuiper-Goodman, 2004; Garcia-Cela *et al.*, 2012). Risk characterization involves a comparison of levels of daily exposure over a lifetime to the TDI to determine whether estimated dietary exposures to mycotoxins are within safe levels. Since the TDI is related to lifetime exposure, it provides a margin of safety that is large enough to allow occasional short-term exposure above the TDI. Nowadays, the bench mark approach, also called margin of exposure (MOE) approach, is considered the most straightforward method for risk characterization of genotoxic and carcinogenic food contaminants according to JECFA and EFSA. Conventionally, dietary exposures to chemicals with a MOE value for oral exposure of 10,000 and above are of low concern from a public health point of view and are considered a low priority for risk management actions The MOE is calculated dividing the $BMDL_{10}$ by the estimated exposure of the mycotoxin in humans. The $BMDL_{10}$ corresponds to the dose at which the incidence of an effect is likely to be smaller than 10% (Kuiper-Goodman, 2004; EFSA, 2007, 2009; Pratt *et al.*, 2009; Garcia-Cela *et al.*, 2012).

The concept of "appropriate level of protection" (ALOP), known as "acceptable level of risk", is also used for risk characterization and further selection of risk management options. In the mycotoxin context, the ALOP comprises two main approaches (FAO/WHO, 2006; Garcia-Cela *et al.*, 2012):

**Notional zero risk approach**: in which hazards are kept at levels that equate to a pre-determined negligible risk indicating that such low exposure levels are reasonably certain not to cause harm. This approach is applied to most mycotoxins and does not produce precise estimates of risk versus dose and cannot model the impact of various interventions in terms of risk reduction.

**Threshold approach:** in which risks must be kept below a specific numerical level as pre-determined by public policy, considered as a "virtually safe dose". This is used for genotoxic carcinogens (i.e. aflatoxins) and the dose-response relationship allows the monitoring of intervention for risk reduction.

# CHAPTER 2

## Determinants of child malnutrition in rural and urban Ecuadorian highlands

*Redrafted after:*

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# 2. Determinants of child malnutrition in rural and urban Ecuadorian highlands

**Abstract**

In this chapter, the socio-demographic determinants of stunting, wasting and overweight amongst children of urban and rural areas in the Ecuadorian highlands were identified and compared. A cross-sectional study (2008) was conducted in a rural (Nabon) and urban (Cuenca) canton of Azuay province, Ecuador. Information of a total of 703 children aged 0-23 months and their caregivers (227 rural and 476 urban) was collected. Stunting prevalence was significantly higher in the rural area (37.4% vs. 17.7%, $P<0.001$) while wasting (7.1%) and overweight (12.4% urban vs. 8.8% rural, $P=0.309$) prevalence were more similar between areas. Determinants of stunting for the pooled sample were male gender (OR=1.43; 95% CI:1.06, 1.92; $P=0.02$), preterm delivery (OR=1.65; 95% CI: 1.14, 2.38; $P=0.008$), child's age (OR=1.04; 95% CI: 1.01, 1.07; $P=0.011$), maternal education (OR=0.95; 95% CI: 0.92, 0.99; $P=0.025$) and facility-based delivery (OR=0.57; 95% CI: 0.45, 0.74; P<0.001). The latter was also a determinant of overweight (OR=0.39; 95% CI: 0.25, 0.62; P<0.001). Rural determinants of stunting were maternal height (OR=0.004; 95% CI: 0.00004, 0.39; $P=0.018$), diarrhea prevalence (OR=2.18; 95% CI: 1.13, 4.21; $P=0.02$), SES (OR=0.79; 95% CI: 0.64, 0.98; $P=0.030$) and child's age (OR=1.07; 95% CI: 1.02, 1.11; $P=0.005$). Urban determinants were: maternal BMI for stunting (OR=0.91; 95% CI: 0.84, 0.99; $P=0.027$), cough prevalence (OR=0.57; 95% CI: 0.34, 0.96; $P=0.036$) and facility-based delivery (OR=0.25; 95% CI: 0.09, 0.73; $P=0.011$) for overweight, and hygiene for wasting (OR=0.57; 95% CI: 0.36, 0.89; $P=0.013$). The socio-demographic determinants associated with infant malnutrition were different between urban and rural areas in Ecuadorian highlands which contribute to prioritize the determinants to be assessed in nutritional interventions.

## 2.1. Introduction

Malnutrition is a primary cause of child mortality and morbidity in developing countries, particularly during the first 5 years of life. Amongst different forms of malnutrition, child stunting (low length-for-age) and wasting (low weight-for-height) remain important public health problems (Black *et al.*, 2008). Stunting is a multi-causal problem occurring as a result of a cumulative process of growth retardation, which does not only depend on temporary food shortages (Smith *et al.*, 2005; Victora *et al.*, 2008), but is also associated with socio-economic, hygiene and other nutritional factors (Grantham-McGregor *et al.*, 2007; Walker *et al.*, 2007; Black *et al.*, 2008). Additionally, maternal characteristics are associated with the risk of child malnutrition. Examples include the negative association between maternal height and child stunting (Bove *et al.*, 2012) and the relation between maternal nutritional status on intrauterine growth restriction and low birth weight (Walker *et al.*, 2007; Black *et al.*, 2008). During the first 2 years of life, children are highly vulnerable to become stunted leading to irreversible development deficiencies towards adulthood (Black *et al.*, 2008; Horton, 2008). These include short-term consequences like child morbidity or disability, immediate consequences like child mortality or long-term consequences like shorter adult height, lower attained schooling, reduced economic productivity, and, for females, lower offspring birth weight (Walker *et al.*, 2007; Black *et al.*, 2008; Victora *et al.*, 2008). Wasting is considered as a better predictor of child mortality than stunting since it is an indicator of the acute nutritional status, generally associated with failure to gain weight or recent weight loss mainly related to acute diseases and acute food shortage (Black *et al.*, 2008).

More recently, the rate of overnutrition in all age groups is also on the increase in developing countries. More problematically, its coexistence with undernutrition has led to the concept of the double burden of malnutrition (Monteiro *et al.*, 2002; Armstrong *et al.*, 2003) which offers important challenges to public health nutrition policies. Moreover, the rapid urbanization phenomenon particularly in Latin America has led to a larger heterogeneity of poverty and malnutrition, scaling up the need for more recent information to build evidence for the formulation of effective interventions and policies adapted to rural and urban areas (Ruel, 2000; Larrea and Freire, 2002; Smith *et al.*, 2005; Hatt and Waters, 2006; Bhutta *et al.*, 2008; Horton, 2008).

According to the Human Development Index, Ecuador is one of the least developed countries in South America together with Bolivia, Colombia and Paraguay (United-Nations-Development-Programme, 2013). From a national representative survey conducted in 2004, high rates of stunting in the first two years of life were reported: 3.2% for children under 5 months, 9.8% for children of 6–11 months and 28.4% for children 12-23 months of age. In contrast, rather low rates of wasting were described: 2.1% for children under 5 months, 3% for children of 6–11 months and 4.6% for children 12-23 months of age; while child overweight prevalence were: 5.3% for

children under 5 months, 3.9% for children of 6-11 months and 4.1% for children of 12-23 months of age (World-Bank, 2007). Ecuador has a rich cultural diversity as well as social, regional and ethnic inequities. Although it is known that poverty and malnutrition affect mostly rural areas and indigenous households (Hall and Patrinos, 2006; World-Bank, 2007), an integral assessment of determinants of malnutrition (i.e. stunting, wasting and overweight) must also comprise the large urban inequities.

This chapter presents the part of the study which aimed at identifying and comparing the socio-economic, health-related, hygiene and nutritional determinants of stunting, wasting and overweight amongst infants and young children of urban and rural areas in the Ecuadorian highlands.

## 2.2. Methodology

### 2.2.1. Setting and survey design

A cross-sectional study was conducted from June to September 2008 in a rural (Nabon) and urban (Cuenca) canton of Azuay province, in the southern Ecuadorian highlands.

Cuenca is the third largest city in Ecuador and is the province capital. Cuenca is located at approximately 2,550 meters above sea level and it has the highest population density in the province with 2% of indigenous population (Guía-Oficial, 2012). Nabon is located in the country side, at 3,000 meters above sea level and at 70 km from Cuenca. Nabon has a considerable territorial dispersion, which makes it difficult to access the different communities. Most of the inhabitants of Nabon (93%) live in the rural areas and 35% consider themselves as indigenous population (Gobierno-Autónomo-Decentralizado-Municipal, 2012). From all Ecuadorian indigenous communities, the Quechua group (34%) is the most predominant in Azuay province (INEC, 2001).

### 2.2.2. Participants (study size, methods of selection)

The study sample consisted of children aged 0 to 23 months and their caregivers. In Nabon, the visited households were randomly selected from the census register of the children under 24 months from all communities of the canton handled by the local workers of the National Institute of Childhood and Family (INFA). In Cuenca, there was no such child register available to be used as sampling frame. Therefore, a cluster random sampling scheme was adopted to select the households. Since the urban plan represents a strict symmetric division in blocks, residential blocks were used as primary sampling unit because of the convenience of selection. The sample size was calculated for both settings with the aim of: i) estimating the prevalence of stunting with a precision of 5% and a confidence interval of 95%, ii) detecting a difference of 15% in the prevalence of stunting between the rural and urban area with statistical power of 90%, type I error of 5%. For both calculations an average stunting prevalence of 30% (World-Bank, 2007) and a non-response of 20% were assumed. For Nabon, this resulted

in a minimal sample size of 232 participants taking into account a correction for finite population (estimated population size was 400 children). For Cuenca, a minimal sample size of 465 participants was necessary assuming an intra-cluster coefficient of 0.2 and an average cluster size of 2 children per block. From this calculation, it was needed to sample 230 blocks. However, during the sampling procedure it became obvious that the average cluster size was overestimated and this was closer to a mean of 1.5 children per block. Therefore, after the first week of surveying, it was decided to sample 400 blocks from a total of 3,111 blocks by random selection to satisfy the proposed level of precision. All households belonging to a selected block were visited door-to-door and the surveys were conducted in households with children under 24 months of age without restriction in the number of children that could be found per block. In every household the youngest child that met the inclusion criteria was selected.

Data were collected by trained medical students by face-to-face interview of child primary caregivers at their homes.

### 2.2.3. Ethics

The study was approved by the Ethical Committee of the University Hospital of Ghent, Belgium, and the Ethical Committee of the Central University of Quito, Ecuador. Prior to the interview, caregivers received an explanation of the study goal and methodology, and written informed consents were obtained from those willing to participate.

### 2.2.4. Survey instrument

The collection of household key indicators was based on the Knowledge-Practices-Coverage (KPC) Rapid Core Assessment Tool on Child Health (CATCH) survey. This instrument also assessed child morbidity: diarrhea defined by three loose stools per day, presence of blood in the stool, persistent cough, difficult breathing, edema, fever and convulsions (Swindale and Bilinsky, 2006). In addition, questions regarding hand washing practices and child care practices during feeding were composed from 3 focus groups with 10 participants each to discuss the practices of infant care. Socio-economic characteristics were assessed using the questionnaire on Unsatisfied Basic Needs (UBN), which was developed by the Integrated Social Indicator System for Ecuador (SIISE, 2007) following the World Bank recommendations (Grosh and Glewwe, 2000). This approach defines a household as “poor” when at least one of the 10 deprivations related to education, health, nutrition, housing, public services and employment opportunities is present, and as “better-off” when no deprivation is reported (Ochoa-Aviles *et al.*, 2012).

The full questionnaire was pre-tested in a convenience sample of 100 households with children under 24 months from childcare centers in a neighboring canton (Azogues, Cañar province). To check for repeatability, the questionnaires were applied in 2 sessions within an interval of 3 weeks. A good correlation was observed between the

first and second application of the surveys (Pearson coeff. = 0.76).

### 2.2.5. Anthropometry measurements and growth characteristics

Anthropometric measurements of both infant and mother were taken in duplicate by teams of two trained data collectors. Birth weight was recorded from parental recall or birth certificate. Following the method described in the Anthropometric Indicators Measurement Guide FANTA (Cogill, 2003), maternal and infant weight was measured with an accuracy of 0.1 kg by using an electronic scale SECA model 803 (Hanover, USA); maternal height was measured using a stadiometer HEALTH-O-METER model portrod (Alsip, USA), recumbent infant length was measured with a length board SECA model 210 (Hanover, USA) and infant mid-upper arm circumference (MUAC) was measured using a non-stretchable measuring tape SECA model 201 (Hanover, USA). Following the method described in MGRS measurement and standardization protocol (De Onis *et al.*, 2004), infant head circumference was measured using a calibrated metric tape SECA model 201 (Hanover, USA). All length measurements were taken with an accuracy of 0.1 cm.

### 2.2.6. Data analysis

Z-scores for length-for-age (LAZ), weight-for-age (WAZ), weight-for-length (WLZ), and BMI-for-age (BMIZ) were calculated using the WHO 2006 growth standard references (WHO, 2006b). Child stunting was defined by a LAZ < -2SD, wasting by a WHZ < -2SD and overweight by a BMIZ > 2SD. Mothers were classified as underweight (BMI < 18.5 kg/m$^2$), normal (BMI: 18.5-24.9 kg/m$^2$), overweight (BMI: 25-29.9 kg/m$^2$), and obese (BMI ≥ 30.0 kg/m$^2$), following the WHO criteria (WHO, 2000).

First, descriptive analyses to compare the characteristics between the urban and rural study samples were presented. For this purpose, chi-square tests in case of categorical variables and student *t*-test for continuous variables were used. Then, the associations between candidate determinants and stunting, wasting, overweight and the continuous variables LAZ, WLZ, BMIZ scores were assessed. This was done first by explorative bivariate analysis. Each determinant with a modest association with one of the outcomes (P-value<0.20) was a candidate to be included in a multivariate regression model. Regression analysis was conducted first using the pooled data, after which the analysis was stratified by study site (rural and urban). Regression models were compiled starting from a saturated regression model using a manual backward elimination strategy removing non-significant predictors based on a Wald test ($P$>0.05). Using this strategy, a logistic regression model was used for the binary outcomes of stunting (LAZ < -2SD), wasting (WLZ < -2SD) and overweight (BMIZ > 2SD), while a linear regression model was adopted for the continuous indices LAZ, WLZ and BMIZ scores.

Because in some cases the mother was absent during the home visit, maternal

anthropometry could not be collected resulting in missing data (24.3%). Nevertheless, to analyze the associations between maternal anthropometry and child nutritional status it was decided to repeat the analysis on a maternal subset of the data with maternal anthropometry available.

Principal component analysis (PCA) with orthogonal rotation was used to compile a proxy index for household socioeconomic status (SES), a care index and a hygiene index. The first PCA factor, which explains the largest possible variation, was used to create these indexes. The SES index was based on: i) house ownership; ii) housing material for wall and floor; iii) toilet type; iv) household source of water; v) number of people that sleep in the caregiver's bedroom; vi) paternal occupation, and vii) maternal occupation. Those variables were selected because they were considered important indicators of the household socio-economic status, and not all of them are part of the key determinants of the UBN classification. Education level and medical attendance were not included in the SES index because it was decided to assess those variables as such, considering their individual importance on child malnutrition. For the SES index the first PCA factor explained 39% of the variance. The index was subsequently divided into quintiles and then grouped as the poor 40%, the middle 40% and the rich 20%. This categorization was chosen in accordance to other studies (Filmer and Pritchett, 2001; Stadskleiv *et al.*, 2008). Because disparities in SES were huge between rural and urban setting, an additional SES per subsample was calculated using the same methodology to assess the association between the distribution of poverty and child nutritional status.

The PCA to create the care index comprised caregiver's information concerning child care practices during feeding: i) whether he/she was younger than 15 or older than 60 years old; ii) whether he/she talked to the child during feeding; iii) whether he/she sat next to the child during feeding; iv) whether he/she taught the child about food during feeding; v) whether the child is allowed to feed him/herself; vi) whether the caregiver let the child eat with his/her own fingers. The first PCA factor explained almost 28% of the variance. The index was divided into tertiles.

The PCA to create the hygiene index included: i) hand-washing practices of the caregiver; whether the caregiver washed his/her hands before food preparation, before feeding children, after defecation and/or after attending to a child who had defecated; ii) hand-washing practices of the child's hands, whether the child's hands were usually washed before feeding and after defecation; iii) the use of soap for hand-washing practices; whether soap was used to wash the caregiver's hands and the child's hands; and iv) source of water for feeding. The first PCA factor explained almost 29% of the variance. The index was divided into tertiles.

All data entry was done in duplicate using EpiData software 3.1 (EpiData Association, Odense, Denmark), which allowed cross-checking double data entry. Data management and analysis were performed in Stata 10.0 (Stata Corporation, College Station, TX). To

account for the clustered sampling in the case of Cuenca, the robust estimation of the standard errors (CLUSTER option in Stata 10.0) was used. Statistical significance was set at $P<0.05$ and all tests were two-sided.

## 2.3. Results

### 2.3.1. Urban-Rural differences

In total 703 children, 476 children from Cuenca (urban area) and 227 children from Nabon (rural area) were included in this study. The sample characteristics of the two settings are presented in Table 2.1. Significantly higher prevalence of stunting (37.4% vs. 17.7%) and severe stunting (18.9% vs. 5.9%) amongst children of the rural area were observed. No significant differences in the prevalence of wasting and overweight were observed. Urban mothers were on average slightly older. Rate of facility-based deliveries was significantly higher in the urban area, where most mothers preferred to attend medical centers instead of traditional healers. No important differences in maternal nutritional status between study samples were observed. However, rural mothers were significantly smaller. Breastfeeding period, either exclusive at 6 months or partial breastfeeding was significantly longer in the rural area. SES determined by PCA and by the UBN indicator showed that Nabon households were significantly poorer (>96%) compared to Cuenca. However, the two methods showed somewhat inconsistent results for Cuenca. According to the UBN approach there were 38.9% poor households, in contrast to 10.7% according to the SES.

In addition, a total of 530 children, 336 children from Cuenca and 194 children from Nabon were included for the maternal subset analysis. No significant difference in the predictors between the pooled and maternal subset was observed.

**Table 2.1.** Main characteristics of the respondents according their living area: Cuenca (urban area) and Nabon (rural area).

| Characteristics | Cuenca [a] | | | Nabon [b] | | | *P* |
|---|---|---|---|---|---|---|---|
| | n | % or mean | SD | n | % or mean | SD | |
| Child's age (months) | 476 | 12.9 | 6.4 | 227 | 13 | 6.5 | 0.894 |
| 0-5 months | | 17.9 | | | 18.1 | | |
| 6-11 months | | 27.1 | | | 24.2 | | |
| 12-17 months | | 28.1 | | | 31.7 | | |
| 18-23 months | | 26.9 | | | 26 | | |
| Male child | 476 | 50.2 | | 227 | 51.5 | | 0.844 |
| Birth weight (grams) [c] | 359 | 3149.2 | 695.2 | 123 | 2996.1 | 736.1 | 0.588 |
| Low birth weight ( ≤2500 g) | 359 | 14.2 | | 123 | 22.8 | | 0.027 |
| Preterm delivery [c] | 476 | 14.3 | | 227 | 17.6 | | 0.323 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Facility-based deliveries | 476 | 96.6 | | 227 | 53.7 | | <0.001 |
| Length-for-age Z-score | 476 | -0.7 | 1.8 | 227 | -1.44 | 1.7 | 0.096 |
| Stunted (<-2SD) | | 17.7 | | | 37.4 | | <0.001 |
| Severely stunted (<-3SD) | | 5.9 | | | 18.9 | | <0.001 |
| Weight-for-length Z-score | 476 | 0.6 | 2.2 | 227 | 0.39 | 2.2 | 0.832 |
| Wasted (<-2SD) | | 7.1 | | | 7.1 | | 0.964 |
| Severely wasted (<-3SD) | | 3.4 | | | 6.2 | | 0.085 |
| Weight-for-age Z-score | 478 | 0.02 | 1.3 | 227 | -0.5 | 1.6 | <0.001 |
| Underweight (<-2SD) | | 5.7 | | | 14.5 | | <0.001 |
| Severe underweight (<-3SD) | | 3.1 | | | 6.2 | | 0.060 |
| BMIZ score | 476 | 0.64 | 2.1 | 227 | 0.51 | 2.2 | 0.825 |
| Overweight (>2-3SD) | | 12.4 | | | 8.8 | | 0.309 |
| Obesity (>3SD) | | 5.7 | | | 6.2 | | |
| Head circumference (cm) | 468 | 45 | 3.3 | 226 | 44.2 | 3.7 | 0.002 |
| MUAC (cm) | 467 | 15 | 1.9 | 226 | 14.4 | 1.7 | 0.448 |
| Child's BMI($kg/m^2$) | 476 | 17.5 | 3.3 | 227 | 17.3 | 3.2 | 0.934 |
| Diarrhea in the last 2 weeks | 476 | 25.4 | | 227 | 21.2 | | 0.468 |
| Cough in the last 2 weeks | 476 | 37.6 | | 227 | 30.4 | | 0.177 |
| Difficult breathing in the last 2 | 476 | 11.1 | | 227 | 10.6 | | 0.892 |
| Fever in the last 2 weeks | 476 | 23.7 | | 227 | 27.8 | | 0.251 |
| Maternal age (years) | 476 | 26.8 | 6.3 | 227 | 25.9 | 7 | 0.083 |
| Maternal height (cm) | 336 | 157.4 | 6.4 | 196 | 152.7 | 7 | <0.001 |
| Maternal weight (kg) | 467 | 59.8 | 9.3 | 218 | 56.5 | 8.6 | 0.077 |
| Maternal BMI($kg/m^2$) | 337 | 24.3 | 3.7 | 194 | 24.2 | 3.7 | 0.999 |
| Underweight | | 4.2 | | | 3.1 | | |
| Normal weight | | 56.1 | | | 62.4 | | |
| Overweight | | 31.8 | | | 29.9 | | |
| Obese | | 8.0 | | | 4.6 | | |
| Maternal school attendance (years) | 476 | 11.5 | 4.2 | 227 | 6.4 | 3.7 | 0.003 |
| No formal education | | 1.3 | | | 11 | | |
| Primary school (1-6 years) | | 12.8 | | | 49.3 | | |
| Secondary school (7-12 years) | | 44.1 | | | 33.5 | | |
| Higher education (≥ 13 years) | | 41.8 | | | 6.2 | | |
| Number of Children <5 years of age (household size) | 476 | 1.4 | 0.6 | 227 | 1.4 | 0.6 | 0.202 |
| Child ever breastfed | 476 | 93.9 | | 227 | 98.2 | | 0.011 |
| Still breastfeeding [d] | 476 | 59.5 | | 227 | 70.5 | | 0.005 |
| Exclusive breastfeeding [e] | 391 | 0.8 | | 186 | 2.2 | | 0.156 |
| <6 months of age | 85 | 61.2 | | 41 | 80.5 | | 0.030 |
| Health seeking behavior | 476 | | | 227 | | | |
| Health service | | 85.1 | | | 76.7 | | 0.006 |
| Traditional healer | | 14.9 | | | 23.4 | | |
| Care index | 476 | | | 227 | | | |
| Low | | 31.1 | | | 36.1 | | 0.407 |

| | | | | |
|---|---|---|---|---|
| Medium | | 33.4 | 30.4 | |
| High | | 35.5 | 33.5 | |
| Hygiene index | 476 | | 227 | |
| Low | | 31.3 | 29.5 | 0.006 |
| Medium | | 27.1 | 47.1 | |
| High | | 41.6 | 23.4 | |
| SES index | 476 | | 227 | |
| Poor 40% | | 10.7 | 97.4 | <0.001 |
| Middle 40% | | 60.3 | 2.2 | |
| Rich 20% | | 29 | 0.4 | |
| UBN | 476 | | 227 | |
| Poor | | 38.9 | 96.5 | <0.001 |
| Better-off | | 61.1 | 3.5 | |

SES, socio-economic status

UBN, Poverty definition based on Unsatisfied Basic Needs

[a] Cuenca's dataset (476 observations).

[b] Nabon's dataset (227 observations).

[c] Birth weight and preterm delivery information was mainly obtained from caregiver recall instead from an official record (83.3% and 93.1%, respectively).

[d] Current breastfeeding, exclusive and not exclusive, at the time of the survey.

[e] Exclusive breastfeeding only for the group of children with age ≥ 6 months (Cuenca n= 391, Nabon n=186).

### 2.3.2. Stunting determinants

Results of the multivariate regression models for length-for-age Z-scores and potential determinants of stunting are presented in Table 2.2. For the pooled dataset, child's age; male gender and preterm delivery were positively associated with stunting whereas facility-based delivery and maternal education were negatively associated with stunting. When analyzing the determinants for both locations separately, no significant determinant of stunting in the urban area was observed; while for the rural area older children and those who had diarrhea episodes in the last 2 weeks prior to the survey were more likely to be stunted. On the other hand, mean LAZ-score for the pooled dataset were negatively associated with child's age and preterm delivery, and positively associated with facility-based deliveries and maternal education. For the urban subsample, mean LAZ-score was negatively associated with child's age and positively associated with maternal education. In Nabon, mean LAZ-score was negatively associated with child's age, and diarrhea episodes and larger household size (larger number of children under 5 years of age in the household).

Additionally, for the maternal subset analysis of the urban area (n=336), children of mothers with higher maternal BMI were less likely to be stunted (OR=0.91; 95% CI: 0.84, 0.99; *P*=0.027). In the rural area (n=194), children of shorter mothers were more likely to be stunted (OR=0.004; 95% CI: 0.00004, 0.39; *P*=0.018), as well as children

from households with a low SES index (OR=0.79; 95% CI: 0.64, 0.98; *P*=0.030); whereas mean LAZ-score was positively associated with maternal height (5.15 LAZ per cm; 95% CI: 2.02, 8.29; *P*=0.001).

**Table 2.2.** Multivariate regression models for LAZ and stunting (linear and logistic regression, respectively) for pooled dataset and in Cuenca (urban area) and Nabon (rural area) separately.

| | LAZ | | | Stunting (LAZ < -2 SD) | | |
|---|---|---|---|---|---|---|
| | Coefficient | 95% CI | *P* | Odds ratio (OR) | 95% CI | *P* |
| **Pooled data** [a] | | | | | | |
| Child's age (months) | -0.06 | -0.08, -0.04 | <0.001 | 1.04 | 1.01, 1.07 | 0.011 |
| Facility-based delivery | 0.57 | 0.35, 0.79 | <0.001 | 0.57 | 0.45, 0.74 | <0.001 |
| Preterm delivery | -0.35 | -0.65, -0.05 | 0.023 | 1.65 | 1.14, 2.38 | 0.008 |
| Maternal education (years) | 0.03 | 0.002, 0.06 | 0.034 | 0.95 | 0.92, 0.99 | 0.025 |
| Male gender | - [d] | - | - | 1.43 | 1.06, 1.92 | 0.020 |
| **Cuenca (urban area)**[b] | | | | | | |
| Child's age (months) | -0.05 | -0.07, -0.03 | <0.001 | - | - | - |
| Maternal education (years) | 0.05 | 0.01, 0.08 | 0.014 | - | - | - |
| **Nabon(rural area)** [c] | | | | | | |
| Child's age (months) | -0.08 | -0.11, -0.05 | <0.001 | 1.07 | 1.02, 1.11 | 0.005 |
| Diarrhea in the last 2 weeks | -0.59 | -1.10, -0.09 | 0.022 | 2.18 | 1.13, 4.21 | 0.020 |
| Number of children <5 years | -0.50 | -0.87, -0.14 | 0.007 | - | | - |

[a] Model adjusted for location (urban vs. rural) for pooled dataset (703 observations)
[b] Cuenca's dataset (476 observations)
[c] Nabon's dataset (227 observations)
[d] (-) no significant association (*P*>0.05)

### 2.3.3. Wasting determinants

Results of the multivariate regression models for weight-for-length Z-scores are presented in Table 2.3. No significant determinant of wasting for the pooled dataset or for the urban/rural subsets was observed. Mean WLZ-score for the pooled dataset was negatively associated with facility-based deliveries and child diarrhea. In the rural area, children that were not delivered in facilities and have ever been breastfed had a higher mean WLZ-score. No significant associations with any of the determinants were

observed for the mean WLZ-score in Cuenca. In the maternal subset, a negative determinant of wasting was hygiene index in Cuenca only (OR=0.57; 95% CI: 0.36, 0.89; *P*=0.013).

**Table 2.3.** Multivariate regression models for WLZ and wasting (linear and logistic regression, respectively) for pooled dataset and in Cuenca (urban area) and Nabon (rural area) separately.

| | WLZ | | |
|---|---|---|---|
| | Coefficient | 95% CI | *P* |
| **Pooled data** [a] | | | |
| Facility-based delivery | -0.77 | -1.04, -0.49 | <0.001 |
| Diarrhea in the last 2 weeks | -0.39 | -0.66, -0.11 | 0.006 |
| **Cuenca (urban area)** [b] | - [d] | - | - |
| **Nabon(rural area)** [c] | | | |
| Facility assisted delivery | -0.71 | -1.28, -0.15 | 0.013 |
| Child ever breastfed | 2.25 | 0.12, 4.37 | 0.038 |

[a] Model adjusted for location (urban vs. rural) for pooled dataset (703 observations)
[b] Cuenca's dataset (476 observations)
[c] Nabon's dataset (227 observations)
[d] (-) no significant association (*P*>0.05)

### 2.3.4. Overweight determinants

Results of the multivariate regression models for BMIZ and overweight (>2SD) are presented in Table 2.4. For the pooled dataset, overweight was less likely for children delivered at facilities. In Cuenca, facility-based delivery was also a negative determinant of overweight and, surprisingly, children with cough episodes seemed to be less likely overweight. In Nabon, children that were not delivered in facilities and have ever been breastfed had a higher mean BMIZ. In the maternal subset of both locations, no maternal-related predictors of overweight were observed.

**Table 2.4.** Multivariate regression models for BMIZ and overweight (linear and logistic regression, respectively) for pooled dataset and in Cuenca (urban area) and Nabon (rural area) separately.

| | BMIZ | | | Overweight (BMIZ > 2 SD) | | |
|---|---|---|---|---|---|---|
| | Coefficient | 95% CI | *P* | Odds ratio (OR) | 95% CI | *P* |
| **Pooled data** [a] | | | | | | |
| Facility-based delivery | -0.76 | -1.04, -0.48 | <0.001 | 0.39 | 0.25, 0.62 | <0.001 |
| **Cuenca (urban area)**[b] | | | | | | |
| Facility-based delivery | - [d] | - | - | 0.25 | 0.09, 0.73 | 0.011 |
| Cough in the last 2 weeks | - | - | - | 0.57 | 0.34, 0.96 | 0.036 |
| **Nabon (rural area)**[c] | | | | | | |
| Facility-based delivery | -0.71 | -1.29, -0.14 | 0.015 | - | - | - |
| Child ever breastfeed | 2.77 | 0.59, 4.95 | 0.013 | - | - | - |

[a] Model adjusted for location (urban vs. rural) for pooled dataset (703 observations)
[b] Cuenca's dataset (476 observations)
[c] Nabon's dataset (227 observations)
[d] (-) no significant association ($P$>0.05)

## 2.4. Discussion

The results demonstrated that stunting is a multi-causal problem influenced by several determinants related to physiological factors, poor access to health services, education, maternal nutritional status, fetal growth and SES. On the other hand, wasting was closely related to inadequate child care. This might be a consequence of access to basic services or education. The results also demonstrate increasing rates of infant overweight and that an important determinant is the poor access to health services. The findings on stunting prevalence are in agreement with the better growth prospects reported for children from urban areas in Ecuador and other Latin American countries (Smith *et al.*, 2005; World-Bank, 2007; Urke *et al.*, 2011). On the other hand, the present results were similar to reported rates for young children in neighboring countries (Maia *et al.*, 2008; Urke *et al.*, 2011; Bove *et al.*, 2012; Martorell and Young, 2012). Then, considerably higher rates of wasting (7.1% vs. 1.8% for rural, and 7.1% vs. 1.6% for urban) and overweight (8.8% vs. 2.8% for rural, and 12.4% vs. 3.5% for urban) than previously

reported in Ecuador but for children under 5 years old (World-Bank, 2007) were observed.

Age and gender are factors that influence individual susceptibility to malnutrition (Wamani *et al.*, 2006; Stadskleiv *et al.*, 2008). Accordingly, in both areas stunting was more prevalent in older children up to 23 months, Typically, mean linear growth is restricted up to the age of 23 months after which a status quo is observed (Victora *et al.*, 2010). Also, boys were more likely to be stunted for the pooled sample, which has been previously observed in other studies (Wamani *et al.*, 2006; Stadskleiv *et al.*, 2008).

According to the UNICEF modified conceptual framework (Shrimpton and Kachondham, 2003), the temporal factors of child malnutrition are related to maternal nutritional status and fetal growth. Prematurity is a common cause of infant mortality and its prevention has been suggested as crucial for the application of stunting management strategies (Espo *et al.*, 2002; Victora *et al.*, 2011). The results also demonstrated the association of preterm delivery with stunting for the pooled sample. Although it might be expected that medical facilities are better in the urban area because of the higher prevalence of prematurity in the rural area, it was observed that prematurity was independent of facility-based delivery. In this study the associations with child birth weight were not assessed due to the considerable proportion of non-responses (31%). A very strong positive association between maternal height and ponderal growth was also found; however, this was found in the rural area only. On the other hand, less child stunting was observed for urban mothers with higher BMI. It was hypothesized that in the urban area other determinants of stunting were more relevant than maternal height due to the population variability and/or maternal height had reached a leveling off status.

In the same framework, child morbidity and inadequate dietary intake are considered immediate causes of child malnutrition. Diarrhea prevalence has been related to acute malnutrition; however repeated episodes can also lead to chronic status of growth faltering, particularly in poor living areas as observed (Ruel, 2000; Espo *et al.*, 2002; World-Bank, 2007). The fact that diarrhea prevalence was related to lower WLZ-scores in Nabon only, might point at a lower and/or more varied hygiene score distribution in this sample. The negative relationship between overweight and cough prevalence was unexpected. However, this relation should be interpreted with caution since the causality cannot be determined in a cross-sectional study. In addition, previous studies described that the relationship between child malnutrition and burden of disease works in both directions (World-Bank, 2007). On the other hand, the benefits of exclusive breastfeeding for infant development and growth are widely acknowledged (Kramer and Kakuma, 2012). Indubitable, breastfeeding practices were better in the rural area. It was also observed that the ever breastfed children had higher WLZ-scores and BMIZ in Nabon only. This observation was not consistent enough because no significant associations were observed when specifying duration and exclusivity of breastfeeding.

Malnutrition can also be related to underlying causes like poor access to health services and inadequate maternal and child care practices (Shrimpton and Kachondham, 2003; Buitrón *et al.*, 2004). Accordingly, the results demonstrate the importance of facility-based deliveries as a protective factor of stunting for the pooled sample, and overweight for the pooled and urban sample. As a direct association is difficult to explain, it is suspected that mothers that delivered in a health facility have a different health seeking behavior that might affect child malnutrition. It was also found that poor hygiene was associated with a higher prevalence of wasting in the urban area only, which might be explained by the more heterogeneous hygienic practices amongst urban households.

Basic causes of child malnutrition, like education and economic resources, are usually more complex and require longer terms to be resolved (Shrimpton and Kachondham, 2003). The very strong impact of maternal education on child malnutrition has been widely described in developing countries, including Latin America (Wamani *et al.*, 2006; Semba *et al.*, 2008; Burchi, 2010; Kac and García Alvear, 2010; Urke *et al.*, 2011; Bove *et al.*, 2012). Lower levels of maternal education were found as a determinant of stunting for the pooled sample with also a higher mean LAZ-score positively associated with maternal education in the urban area only. This finding might be related to the fact that, in urban center, education could be valorized into better job opportunities and therefore leading to considerable higher income. This rural/urban differences of maternal education related to socioeconomic status had also been addressed in some Andean countries (Larrea and Kawachi, 2005; Hatt and Waters, 2006). The strong association between low household economic status and child malnutrition has been reported for several Latin American countries (Smith *et al.*, 2005; Wamani *et al.*, 2006; Semba *et al.*, 2008; Van de Poel *et al.*, 2008; Burchi, 2010; Kac and García Alvear, 2010; Urke *et al.*, 2011; Bove *et al.*, 2012). Although a significant positive crude association between UBN and SES index was observed, and the mean LAZ-score respectively (results not shown), this relation disappeared when the model was adjusted for urban/rural location in the pooled dataset. Only for the maternal subset, it was observed that children from rural households with a low SES index were more likely to be stunted. Those findings suggest strong differences in SES between the urban and rural locations, but smaller differences in associations with child malnutrition within the study subsamples. It is also hypothesized that this is because part of the association is probably captured by more direct determinants of malnutrition which further attenuate the association between child malnutrition and SES proxies (i.e. UBN classification and SES index). One of the proxies of SES related to stunting is the number of children in the household, which influences the resources available to each child, in terms of financial, time and attention (Sereebutra *et al.*, 2006; World-Bank, 2007). It was observed that a larger number of children under 5 years old in the household was related to mean LAZ-score in Nabon only, and this could be seen as a proxy of economical burden and maternal occupational burden. A remarkable finding was the inconsistent results of prevalence of poverty in Cuenca using the UBN method and the SES index. Apparently larger socioeconomic inequalities coexist at urban level.

Then, the inclusion of parental occupation (ranked according to salary) in the construction of the SES index gives an important additional argument to classify and explain better the socioeconomic status at urban level.

A number of limitations need to be addressed. First, mere associations can be described and only non causal relationships could be established due to the cross-sectional design of the study. Second, as the study was questionnaire-based, some questions that required a good memory were vulnerable to recall bias (like birth weight and morbidity in the last 2 weeks); also it cannot be excluded that some questions triggered a social prestige bias (like hygiene or care practices). Third, maternal anthropometry could not be obtained in cases where the mother was not home during the interviews. In such cases, the caregiver was interviewed and it remains unclear to what extent this might have influenced the data quality. However, after testing possible variations between the pooled and maternal subset, no significant difference was found. Fourth, some immediate (nutrient intake) and underlying (food security) determinants of the conceptual framework of child malnutrition were not assessed. Finally, although the determinants of child malnutrition would remain the same, Ecuadorian governmental policies have changed since the data were collected (2008), particularly those regarding better access to health services (SENPLADES, 2009).

## 2.5. Conclusions

In this chapter, the major proxies of child malnutrition identified were presented, i.e. stunting followed by overweight. Being stunting the main child nutritional failure in Ecuador, these results demonstrated persistent and alarming rates since those are as high as nationally reported in 1986, which could protract the goal of 12% by 2015 (World-Bank, 2007). Thus, the intervention strategies must be targeted to the most vulnerable population groups and tackling the most relevant determinants. In this study the socio-demographic and -economical determinants of child malnutrition between children from rural and urban highlands in Ecuador were identified and compared. No common determinants between both settings for any type of malnutrition were observed. For the pooled sample, stunting was more likely amongst male, older and preterm children, as well as children not delivered at facilities and children of mothers who had lower levels of maternal education; whereas overweight was also more likely for children delivered at facilities. Urban determinants were related to maternal nutritional status (maternal BMI) for stunting, child morbidity (cough) and access to health services (facility-based delivery) for overweight, and child care (hygiene index) for wasting. Rural determinants of stunting were related to maternal nutritional status (maternal height), child morbidity (diarrhea) and inherent factors (child's age) and SES; whereas no significant determinants were observed for overweight and wasting. Therefore, it is suggested that intervention strategies must be mainly related to primary health issues and education.

# CHAPTER 3

## Socio-demographic determinants of child feeding patterns in rural and urban Ecuadorian highlands

# 3. Socio-demographic determinants of child feeding patterns in rural and urban Ecuadorian highlands

**Abstract**

In this chapter, a comparison of the child feeding practices between an urban and rural area in the Ecuadorian highlands is presented. Data were collected from a total of 998 children aged 0-23 months and their caregivers (348 rural and 650 urban). A number of the WHO child feeding indicators were assessed. Breastfeeding practices were better in the rural area, whereas the intake of complementary and weaning food was higher in the urban area. The basic diet in both settings was characterized by a high consumption of plant-based foods, including cereals and cereal products (20% urban vs. 26% rural), dairy products (29% urban vs. 18% rural), fruits and vegetables (11% urban vs. 15% rural) and tubers (11% urban vs. 22% rural). In both areas, the consumption of plant-sources of vitamin A and flesh foods (meat, poultry and liver/organ meats) was remarkably low. The differences in average energy and nutrient intake between the urban and rural setting were assessed by adjusting regression models for facility-based delivery; health seeking behavior, maternal age, maternal age at first delivery, maternal education, maternal working status, number of children under 5 years at the household, maternal marital status and additional income from migration were added to the analytical models. The urban-rural difference in breast milk intake remained after adjustment, while those covariates partially explained the urban-rural difference in energy intake from solid/semi-solid foods (52-66%) and in feeding indicators (9-44%). Maternal education and facility-based delivery were the most important socio-demographic covariates that explained the urban-rural differences. Both were defining characteristics in the urban area and this could rely on higher health consciousness and indirectly with better-off household conditions.

## 3.1. Introduction

Child malnutrition, including micronutrient deficiencies, remains one of the main public health challenges of the 21st century particularly in developing countries where detrimental factors related with poverty are commonly faced. Despite several worldwide efforts, child and maternal undernutrition are the underlying cause of 3.1 million deaths and 45% of all child deaths (Grantham-McGregor *et al.*, 2007; Victora *et al.*, 2010; Black *et al.*, 2013).

Among the critical pillars to maintain a good nutritional and health status are proper feeding practices during infancy and early-childhood (Ruel and Menon, 2002; Saha *et al.*, 2008). Globally, the WHO recommendations for appropriate breastfeeding, complementary feeding and feeding for non-breastfed infants are well known (WHO, 2002, 2005). However, their accomplishment can be hampered by a series of interrelated factors (Kamudoni *et al.*, 2007; Senarath *et al.*, 2007). In Ecuador, for instance, it has been suggested that geographical differences could lead to different conditions of food accessibility and food availability, and therefore have an influence on child feeding patterns (Leonard *et al.*, 2000). In this context, the basic diet in the highlands of Ecuador has been described as monotonous which could evolve into macro and micronutrient deficiencies (World-Bank, 2007). Moreover, persisting high rates of child malnutrition have been reported in Ecuador, with stunting as the main nutritional failure in both rural (37.4%) and urban (17.7%) areas (*Chapter 2*). Besides stunted growth, micronutrient deficiencies of iron, zinc and vitamin A have been recognized as primary child nutritional failures amongst Ecuadorian children as well as in most developing countries (WHO/PAHO, 2003; Muller and Krawinkel, 2005; World-Bank, 2007; Lutter *et al.*, 2008).

Urbanization phenomenon has been suggested as important influencing factor of food security and subsequently of feeding patterns in developing countries (Ruel and Menon, 2002; Perez-Escamilla, 2003; Smith *et al.*, 2005; Enneman *et al.*, 2009). Moreover, dietary patterns can differ across different socioeconomic groups. It has been suggested that the underlying socioeconomic and socio-demographic proxies, like occupation and education, can influence on dietary patterns similarly but independently from each other (Galobardes *et al.*, 2001; Turrell *et al.*, 2003; Darmon and Drewnowski, 2008). In the previous chapter it was described that urban determinants were related to maternal BMI for stunting, child morbidity (cough) and facility-based delivery for overweight, and child care (hygiene index) for wasting; whereas rural determinants were related to maternal height, child morbidity (diarrhea), child's age and socioeconomic status (SES) for stunting and no significant determinants were found for overweight and wasting (*Chapter 2*). Nevertheless, a holistic evaluation of child malnutrition must also comprise the assessment of feeding patterns and how these are influenced by urban-rural behaviors, opportunities and disadvantages.

This chapter presents the part of the study that aimed at: i) comparing the energy and nutrient intake of breastfed and non-breastfeed children aged 0-23 months between urban and rural areas in the Ecuadorian highlands; ii) comparing the breastfeeding and complementary feeding practices between urban and rural areas; iii) identifying the key nutrient sources amongst the diets of both settings, and iv) distinguishing the socio-demographic characteristics that explain the urban-rural differences in child feeding patterns.

## 3.2. Methodology

### 3.2.1. Setting and survey design

This section of the study was part of the cross-sectional survey (previously described in *Chapter 2*). Briefly, this survey was conducted from June to September 2008 in a rural (Nabon) and urban (Cuenca) canton located at the southern Ecuadorian highlands. The studied children from the rural setting had a higher prevalence of stunting compared to the urban setting (37.4% vs. 17.7%), a lower prevalence of overweight (8.8% vs. 12.4%) but demonstrated similar prevalence of wasting (7.1%).

### 3.2.2. Participants (study size, methods of selection)

This section of the study was conducted in children aged 0 to 23 months and their caregivers. Data were collected from the primary child caregivers at their homes by face-to-face interviews. The methods of selection of participants were previously described (*Chapter 2*). Briefly, in Nabon, the households were randomly selected from a census register of children aged 0-23 months from all communities of the canton. In Cuenca, the households were selected adopting a cluster random sampling scheme and using residential blocks as primary sampling unit. In each household, information that was collected corresponded to the youngest child that met the inclusion criteria.

The sample size calculation of this survey was based on detecting a difference in stunting prevalence between rural and urban study settings (*Chapter 2*). However, with a sample size of 348 for Nabon and 650 participants for Cuenca a difference of 100 $kcal.d^{-1}$ in energy intake between the urban and rural setting could be detected considering an average energy intake of 355 ± 441 kcal $d^{-1}$ (Leonard *et al.*, 2000).

### 3.2.3. Data collection

#### 3.2.3.1. Demographic characteristics

Data collection of demographic characteristics of this study was previously described (*Chapter 2*). Household indicators were collected based on the Knowledge-Practices-Coverage (KPC) Rapid Core Assessment Tool on Child Health (CATCH) survey (Swindale and Bilinsky, 2006), and socio-economic characteristics were assessed using

the questionnaire on Unsatisfied Basic Needs (UBN) that was developed by the Integrated Social Indicator System for Ecuador (SIISE, 2007) following the World Bank recommendations (Grosh and Glewwe, 2000).

#### 3.2.3.2. Child feeding patterns

Child feeding patterns were assessed using the Knowledge-Practices-Coverage (KPC) survey (Module 2: Breastfeeding and infant and young child feeding) (Dewey *et al.*, 2006), which is a tool to evaluate the ten Guiding Principles for complementary feeding developed by the WHO (WHO/PAHO, 2003). This survey was accordingly adapted to the studied areas during pre-testing.

#### 3.2.3.3. Dietary assessment

The quantity of food consumed was estimated using maternal 24 hours recalls (24-hr). In the urban area, two interactive 24-hr were carried out on non-consecutive days (the first from a weekday and the second from the weekend) with a maximum delay of three weeks between both recalls. In the rural area, only one 24-hr was performed due to constraints in accessibility. This recall was performed in a weekday. To estimate portion sizes, each respondent was asked to fill a household recipient with the actual amount of food consumed by the child. Trained interviewers were equipped with a set of household measures (bottle, plate, cup, spoon and teaspoon) and a measuring jar to determine this amount in mL. To convert this into grams, the consistency of each food was recalled and estimated by comparison with 5 foodstuff models with known densities: i) spongy-solid (e.g. fruit); ii) semi-solid (e.g. porridge), iii) soft (e.g. jelly), iv) semi-soft (e.g. gruel) and liquid (e.g. watery). In addition, detailed recipe data of all composite preparations were recalled from each respondent and then used to calculate the actual amount of each ingredient. Average recipe data were used when the actual recipe was not available (Gibson *et al.*, 2009). The average recipes for each area were composed by the ingredients which were common in more than the 60% of the total of recipes compiled under the same recipe name in each setting.

To assess the breast milk intake, the average length (in minutes) of each suck was asked to the mothers. For the conversion of time estimations to grams of breast milk ingested, a proxy conversion factor of 6.15 grams of milk per minute of breastfeeding was employed. This factor was calculated based on the mean intake of human milk (g d-1) in developing countries of infants from 1 to 12 months of age (Butte et al., 2002), an average number of 10 sucks per day and an average length of 10 minutes per suck. These average values were calculated from the information gathered in this study.

As indicated in *Chapter 2*, all questionnaires were pre-tested and checked for repeatability in a convenience sample of 100 households with children aged 0-23 months who attended childcare centers in a neighboring canton. A good correlation was observed between the first and second interactive 24-hr procedure within an interval of

3 weeks (Pearson coeff. = 0.99).

#### 3.2.3.4. Food data sources

The main dietary sources for each nutrient and energy content were identified. The food group-system used was based on the WHO food grouping for dietary diversity assessment (7 groups) (WHO, 2008), to which 3 subgroups were added to adapt to the Ecuadorian context: i) cereals and cereal products (alone); ii) fats and fatty snacks, and iii) infants foods (formula and fortified baby feeding powder mixtures). Fruits and vegetables rich in vitamin A were defined using a cut-off of >130 µg RE (retinol equivalents) per 100 g (Dewey *et al.*, 2006). Meat, poultry and liver/organ meats were designated as "flesh" foods.

Data entry was done in duplicate using EpiData software 3.1 (EpiData Association, Odense, Denmark), with the exception of the 24-hr data for which the Lucille food intake software® was used (Ghent-University, 2010). This software allowed the quantitative estimation of food intake at nutrient level based on pre-set food composition databases. Due to the lack of a reliable Ecuadorian food composition data, a food composition table was compiled using mainly Peruvian (Bejarano *et al.*, 2002; Reyes *et al.*, 2009) and Costa Rican data (INCAP/OPS, 2012). Information of very few food items were also gathered from Mexican (INNSZ, 1999) and Chilean data (Jury *et al.*, 1997). In addition, for a number of traditional foods, laboratory analyses were performed. Representative food samples from the main local markets and supermarkets in Cuenca were collected following the sampling plan recommendations given by INFOODS (International Network of Food Data Systems) (Greenfield and Southgate, 2003). Composite samples were analyzed, in triplicate, determining the content of: moisture and dry matter (by desiccation), ash (by calcination), total fat (by Weibull), total protein (by Kjeldahl, based on total nitrogen and using the conversion factor given by (FAO/WHO, 1973), total carbohydrates (by difference) and energy content (Atwater conversion factors) according to the analytical methods suggested by INFOODS (Greenfield and Southgate, 2003). Micronutrient content data were obtained from the USDA database (USDA, 2013b).

### 3.2.4. Data analysis

A number of indicators for infant and young child feeding practices suggested by WHO (WHO, 2008) were assessed. Children were classified into exclusively breastfed, predominantly breastfed, complementary fed and non-breastfed (weaning) following the criteria of WHO guidelines (WHO, 2008). Means of sample characteristics and infant feeding indicators between the urban and rural setting were first compared using bivariate analysis. To account for the clustered sampling design in Cuenca, a linear regression model was used with the characteristic as outcome and setting as a predictor. For categorical variables, for the same reason, a logistic regression model was used.

Besides the socio-economic classification based on Unsatisfied Basic Needs, a proxy index for household socio-economic status was created by using principal component analysis (PCA), as previously described (*Chapter 2*).

Descriptive analyses for estimated daily energy and nutrient intakes (median and interquartile distances) for breastfed and non-breastfed children of 6-8 months, 9-11 months and 12-23 months of age were performed according to setting. For breastfed children, the contributions from breast milk and complementary food were estimated. In the urban area, individual intake was based on 2 days of dietary recall (mean), while in the in the rural area it was based on one 24-hr. The median daily intakes were compared with the dietary reference intake (DRI) for breastfed and non-breastfed children aged 6-8 months, 9-11 months and 12-23 months (WHO, 1998; Dewey and Brown, 2003; WHO/PAHO, 2003; WHO, 2005; Dewey *et al.*, 2006).

Differences in food and nutrient intakes between urban-rural settings were analyzed adjusting for age and gender imbalances between settings because these characteristics were not expected to depend on setting. Thereafter, the analysis for differences in socio-demographic covariates was adjusted to asses to what extent the crude differences between settings can be explained by such covariates. A similar analysis was adopted to compare the feeding indicators between the urban and rural setting.

Data management and analysis were performed in Stata 10.0 (Stata Corporation, College Station, TX). For all analyses a robust estimation of the variance was adopted using the cluster option in Stata to adjust for the clustered sampling design in Cuenca. All tests were two-sided and statistical significance was set at $P<0.05$.

## 3.3. Results

### 3.3.1. Urban-Rural differences

In total, 998 infants, 650 from Cuenca (urban area) and 348 from Nabon (rural area) were included in this part of the study. The sample characteristics of both settings are presented in Table 3.1. The proportion of facility-based deliveries in Cuenca was approximately twice of that in the rural area (96.3% vs 50.3%; $P<0.001$). However, traditional therapies were preferred more in the rural community than in the urban area (24.8% vs 15.2%; $P<0.001$). Cough episodes in the last 2 weeks prior to the survey were more frequently reported in the urban area (37.7% vs 29.3%; $P<0.001$), while more fever episodes were reported in the rural area (26.4% vs 22%; $P=0.014$).

Maternal school attendance was higher in the urban area (11.5 vs 6.4 years; $P<0.001$) and higher rates of illiteracy were observed in the rural area (10.8% vs 1.0%). Better socio-economic proxies were observed in the urban area. Working outside home was more common among urban mothers (51.9% vs 40.7%; $P<0.001$). Household size (number of children under 5 years old) was larger in rural households (1.5 vs 1.4;

*P*=0.014). A lower prevalence of single-parent households was observed in the urban area (16.3% vs 27.3%; *P*<0.001). The prevalence of receiving extra incomes from migration was higher among urban households (14.2% vs 7.2%; *P*<0.001). Most of the rural households were classified as poor (>96%) based on SES index and the UBN system. However, the prevalence of urban households classified as poor differed between both approaches (11.1% according to the SES index vs. 37.9% according to UBN).

**Table 3.1.** Main characteristics of the participants according their living area: children aged 0-23 months and their caregivers (n=998), Cuenca (urban area, n=650) and Nabon (rural area, n=348).

| | **Cuenca** | | | **Nabon** | | | |
|---|---|---|---|---|---|---|---|
| Characteristics | n | % or mean | SD | n | % or mean | SD | *P* [a] |
| Child's age (months) | 650 | 12.1 | 6.7 | 348 | 12.0 | 7.0 | 0.588 |
| 0-5 months | | 23.5 | | | 26.7 | | |
| 6-11 months | | 25.4 | | | 23.6 | | |
| 12-17 months | | 26.3 | | | 25.6 | | |
| 18-24 months | | 24.8 | | | 24.1 | | |
| Male child | 650 | 51.9 | | 348 | 49.1 | | 0.165 |
| Facility-based deliveries | 625 | 96.3 | | 330 | 50.3 | | <0.001 |
| Health seeking behavior | 645 | | | 347 | | | <0.001 |
| Health service | | 84.8 | | | 75.2 | | |
| Traditional healer | | 15.2 | | | 24.8 | | |
| Diarrhea in the last 2 weeks | 650 | 24.5 | | 348 | 21.0 | | 0.052 |
| Cough in the last 2 weeks | 650 | 37.7 | | 348 | 29.3 | | <0.001 |
| Difficult breathing in the last 2 weeks | 650 | 10.6 | | 348 | 10.6 | | 0.989 |
| Fever in the last 2 weeks | 650 | 22.0 | | 348 | 26.4 | | 0.014 |
| Maternal age (years) | 646 | 26.6 | 6.5 | 346 | 26.1 | 7.2 | 0.189 |
| Maternal age at first delivery (years) | 638 | 21.5 | 4.7 | 339 | 19.9 | 3.7 | 0.149 |
| Maternal school attendance (years) | 627 | 11.5 | 4.2 | 316 | 6.4 | 3.7 | <0.001 |
| No formal education | | 1.0 | | | 10.8 | | |
| Primary school (1-6 years) | | 12.4 | | | 47.8 | | |
| Secondary school (7-12 years) | | 43.5 | | | 36.1 | | |
| Higher education (≥ 13 years) | | 43.1 | | | 5.4 | | |
| Maternal working status | 632 | | | 339 | | | |
| Working outside home | | 51.9 | | | 40.7 | | <0.001 |
| Number of children <5 years of age (household size) | 650 | 1.4 | 0.6 | 348 | 1.5 | 0.6 | 0.014 |
| Maternal marital status | 649 | | | 344 | | | |
| Single-parent household | | 16.3 | | | 27.6 | | <0.001 |

| | | | | | |
|---|---|---|---|---|---|
| Additional income from migration | 640 | 14.2 | 348 | 7.2 | <0.001 |
| SES index | 623 | | 321 | | |
| Poor 40% | | 11.1 | | 96.9 | <0.001 |
| Middle 40% | | 59.1 | | 2.8 | |
| Rich 20% | | 29.9 | | 0.3 | |
| UBN | 650 | | 348 | | |
| Poor | | 37.9 | | 96.0 | <0.001 |
| Better-off | | 62.1 | | 4.0 | |

SES, socio-economic status by using principal component analysis (PCA).
UBN, poverty definition based on Unsatisfied Basic Needs questionnaire.
[a] A linear regression model was used for continuous variables and a logistic regression model for categorical variables, with robust estimation of the variance to account for the clustered sampling scheme in Cuenca.

### 3.3.2. Infant feeding practices

The feeding indicators of both settings, as well as the crude and adjusted analyses of the urban-rural difference, are presented in Table 3.2. In general, although the high prevalence of "ever" breastfed children in both settings (≥ 94%), better breastfeeding practices were observed in the rural area. Particularly, the prevalence of exclusive breastfeeding was higher for rural infants (59% vs. 19%, $P<0.001$). Early initiation of breastfeeding (within one hour of birth) was also more frequent in the rural area (92% vs. 82%; $P<0.001$). Moreover, continued breastfeeding after one year was higher for rural infants compared to those in the urban area (81% vs. 58%, $P<0.001$). Similarly, children were longer breastfed (total duration) in Nabon compared to Cuenca (12.2 vs. 9.5 months; $P<0.001$). Although a high prevalence of introduction of solid/semi-solid foods at 6-8 months were observed in both areas, this was less the case in the Nabon (89% vs. 96%, $P=0.007$). In infants younger than 6 months of age the prevalence of non-timely complementary feeding (50% vs. 20%; $P<0.001$) and weaning (10% vs. 4%; $P<0.001$) were higher in the urban area (data not shown).

The analysis of the urban-rural difference in feeding indicators was adjusted with the socio-demographic characteristics that largely differed between settings. These were facility-based delivery; health seeking behavior, maternal age, maternal age at first delivery, maternal education, maternal working status, household size, maternal marital status and additional income from migration. The selected covariates partially explained the difference by setting (9-44%) on all feeding indicators, with the exception of introduction of solid/semi-solid foods at 6-8 months. When adjusting the analysis for each of the socio-demographic covariates separately, it was observed that for most indicators (early initiation of breastfeeding, ever breastfeeding, total duration of breastfeeding and continued breastfeeding at 2 years old) the covariates that substantially explained the urban-rural difference were facility-based delivery (reduction of 16-39%) and maternal education (reduction of 16-43%). Urban-rural

differences in exclusive breastfeeding, introduction of solid/semi-solid foods at 6-8 months and continued breastfeeding at 1 year old were mostly explained by maternal education (reduction of 19-21%); while predominant breastfeeding was mostly explained by facility-based delivery (reduction of 16%).

Significant associations between the feeding indicators and the socio-demographic covariates were observed. Ever being breastfed was less likely the case amongst infants from single-parent households (OR=0.26; 95% CI: 0.12, 0.54; $P<0.001$) and in those households that received additional income from migration (OR=0.39; 95% CI: 0.18, 0.86; $P=0.020$). Breastfeeding within the first hour after birth was more prevalent for larger household size (OR=1.52; 95% CI: 1.06, 2.17; $P=0.023$). Exclusive breastfeeding was more likely amongst infants delivered at health facilities (OR=2.52; 95% CI: 1.22, 5.21; $P=0.012$). Continued breastfeeding at 2 years was less likely amongst infants from households receiving extra incomes from migration (OR=0.20; 95% CI: 0.08, 0.53; $P=0.001$). The odds of receiving solid/semi-solid food at 6-8 months was higher in children of older mothers (OR=1.15; 95% CI: 1.09, 1.22; $P<0.001$), in mothers with higher education level (OR=1.28; 95% CI: 1.14, 1.43; $P<0.001$) and about ten times higher in children delivered at health facilities (OR=9.93; 95% CI: 1.53, 64.36; $P=0.016$). Surprisingly, this practice was less likely in children of households that preferred professional health attendance (OR=0.35; 95% CI: 0.21, 0.59; $P<0.001$). The total duration of breastfeeding was negatively associated with facility-based delivery (-1.36 months; 95% CI: -2.59, -0.13; $P=0.030$). The age of introduction of solid/semi-solid foods was also negatively associated with facility-based delivery (-0.46 months; 95% CI: -0.78, -0.14; $P=0.005$), as well as with single-parent households (-0.40 months; 95% CI: -0.76, -0.04; $P=0.028$).

**Table 3.2.** Feeding indicators and analysis of the difference and adjusted difference between urban and rural setting: children aged 0-23 months (n=998), Cuenca (urban area, n=650) and Nabon (rural area, n=348).

| | **Cuenca** | **Nabon** | **Difference** [a] | | **Adjusted difference** [b] | |
|---|---|---|---|---|---|---|
| | n (%) | n (%) | OR/effect (95% CI) | *P* | OR/effect (95% CI) | *P* |
| Child ever breastfed (0-23 months) | 611/650 (94) | 339/348 (97) | 3.47 (2.48; 4.86) | <0.001 | 2.43 (1.43; 4.11) | 0.001 |
| Early initiation of breastfeeding (0-23 months) | 496/606 (82) | 311/338 (92) | 2.34 (1.86; 2.94) | <0.001 | 1.80 (1.32; 2.46) | <0.001 |
| Exclusive breastfeeding (0-5 months) | 29/153 (19) | 55/93 (59) | 6.64 (4.05; 10.87) | <0.001 | 5.91 (2.96; 11.81) | <0.001 |
| Predominant breastfeeding (0-5 months) | 31/153(20) | 15/93 (16) | 0.80 (0.52; 1.22) | 0.297 | 0.71 (0.36; 1.42) | 0.336 |
| Continued breastfeeding at 1 year (12-15 months) | 63/109 (58) | 50/62 (81) | 3.20 (2.09; 4.89) | <0.001 | 2.54 (1.35; 4.77) | 0.004 |
| Continued breastfeeding at 2 years (20-23 months) | 19/94 (20) | 17/63 (27) | 1.9 (0.58; 1.69) | 0.986 | 0.32 (0.12; 0.83) | 0.019 |
| Introduction of solid/semi-solid foods (6-8 months) | 82/85 (96) | 34/38 (89) | 0.19 (0.05; 0.64) | 0.007 | 0.32 (0.01; 8.73) | 0.499 |
| Total duration of breastfeeding (months) | 9.5±5.4[c] | 12.2±5.5[c] | 2.17 (1.37; 2.98)[d] | <0.001 | 1.22 (0.10; 2.35)[d] | 0.033 |
| Age of introduction of solid/semi-solid foods (months) | 5.6±2.4[c] | 5.8±2.0[c] | 0.12 (-0.08; 0.32)[d] | 0.243 | -0.24 (-0.55; 0.07)[d] | 0.126 |

[a] From a logistic regression model adjusted for child's sex and age and adjusted for clustered sampling design in Cuenca

[b] From a logistic regression model adjusted for child's sex and age, facility-based delivery; health seeking behavior, maternal age (years), maternal age at first delivery (years), maternal education (years), maternal working status, number of children <5 years, maternal marital status and additional income, and adjusted for clustered sampling design in Cuenca from migration.

[c] mean ±SD

[d] Estimates (95% CI) were presented for continuous variables.

### 3.3.3. Food and nutrient intakes

The energy contribution from breast milk and solid/semi-solid foods at different age stages in Cuenca and Nabon was compared in Figure 3.1. For infants below 6 months, the total energy intake was similar in both settings. Thereafter, a gradual increase was only observed for children of the urban area. Although the energy contribution of breast milk was considerably higher in the rural area, the difference in total energy intake was particularly attributable to the higher consumption of solid/semi-solid foods in the urban area.

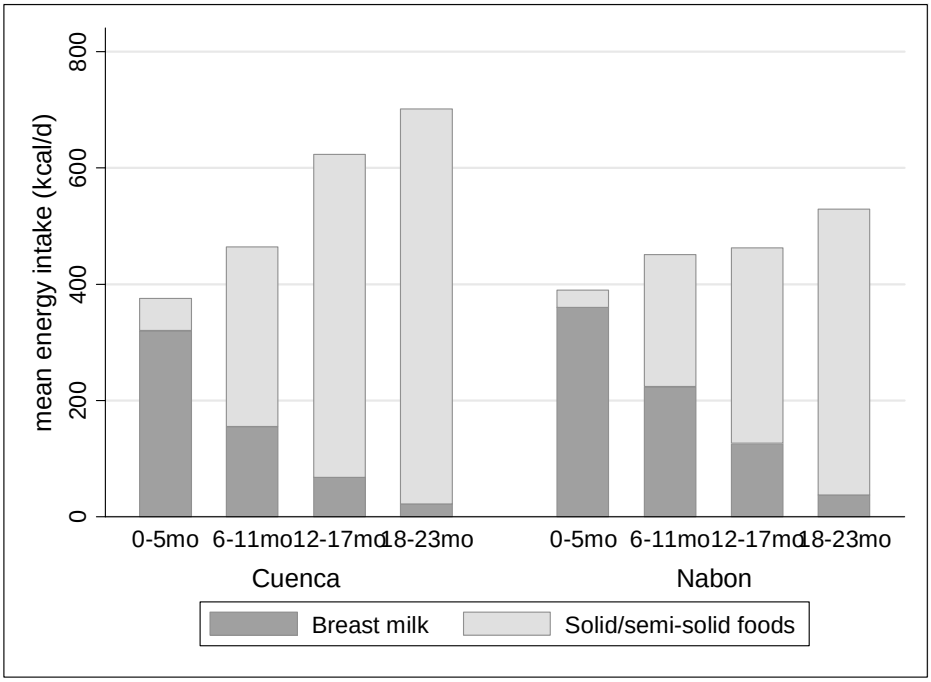


**Figure 3.1.** Energy contribution from complementary foods and breast milk according to age and setting: children aged 0-23 months (n=998), Cuenca (urban area, n=650) and Nabon (rural area, n=348).

The median dietary intakes for breastfed and non-breastfed children older than 6 months at three different age groups are presented in Table 3.3. In the rural area, the breast milk intake was considerably higher for children at 6-8 months ($P<0.001$), at 9-11 months ($P=0.020$) and 12-23 months ($P<0.001$). In contrast, the energy intake of solid/semi-solid foods in the urban area was higher for breastfed at 6-8 months ($P<0.001$), at 9-11 months ($P=0.002$) and at 12-23 months ($P<0.001$). Similarly, the energy intake of solid/semi-solid foods in the urban area was higher for non-breastfed children at 9-11 months ($P=0.001$) and at 12-23 months ($P<0.001$) (data not shown). The median energy intake (and even the 75$^{th}$ percentile value) from breast milk were found suboptimal for both rural and urban infants when comparing with the DRIs for breast milk (413 kcal at 6-8 months, 379 kcal at 9-12 months and 346 kcal at 12-23 months)

(WHO/PAHO, 2003; Dewey *et al.*, 2006).

Similarly, the median energy intakes from solid/semi-solid foods at complementary and weaning stage were suboptimal in both settings, except for breastfed children at 6-8 months in Cuenca and non-breastfed children at the same age in Nabon. However, when considering the 75$^{th}$ percentile value of energy intake, the compliance with the DRIs was higher in the urban area. Median protein intakes from solid/semi-solid foods were higher than the DRIs in the urban area and with few exceptions in the rural area. The mean percentage of energy consumed as fat in complementary foods was usually higher than recommended, except for breastfed and non-breastfed children aged 12-23 months in Nabon. Median micronutrient intakes were lower than the DRIs, except for the median intake of vitamin A and zinc for non-breastfed children at 6-8 months in the rural area. When considering the 75$^{th}$ percentiles, infants of the urban area had a higher compliance with the DRIs for micronutrients.

**Table 3.3.** Daily energy and nutrient intakes, expressed as median (25th and 75th percentiles), for breastfed and non-breastfed children according to setting: children aged 6-23 months and their caregivers (n=752), Cuenca (urban area, n=497) and Nabon (rural area, n=255).

| | | | **Cuenca** | | | **Nabon** | | |
|---|---|---|---|---|---|---|---|---|
| | DRI (total)[a] | DRI (CF)[b] | Breastfed children (n=261) | | Non-breastfed children (n=236) | Breastfed children (n=166) | | Non-breastfed children (n=89) |
| | | | Breast milk | solid/semi-solid foods | | Breast milk | solid/semi-solid foods | |
| **6-8 months** | | | | | | | | |
| Energy (kcal) | **615** | **202** | 159 (86, 269) | 219 (103, 321) | 306 (127, 505) | 215 (129, 400) | 97 (11, 155) | 817 (449, 879) |
| Energy from fat (%) [c] | **30%** | **0%** | - | 21% | 28% | - | 11% | 27% |
| Proteins (g) | **9.6** | **2** | 2.3 (1.3, 4.0) | 5.4 (1.7, 9.0) | 10.1 (2.6, 14.3) | 3.2 (1.9, 5.9) | 0.8 (0.04, 3.9) | 24.0 (13.1, 43.5) |
| Vitamin A (µg RE) | **500** | **63** | 139 (75, 234) | 52 (15, 106) | 87 (19, 245) | 188 (113, 349) | 6 (0, 38) | 537 (186, 660) |
| Iron (mg) | **11** | **9.1** | 0.07 (0.04,0.1) | 1.7 (0.8, 2.9 ) | 2.0 (1.3, 3.3) | 0.09 (0.05, 0.17) | 0.4 (0.1, 1.1) | 2.5 (0.6, 3.3) |
| Zinc (mg) | **3** | **2.2** | 0.4 (0.2,0.7) | 0.9 (0.3, 1.1) | 1.0 (0.7, 1.8) | 0.5 (0.3, 1.0) | 0.3 (0.06, 0.7) | 3.2 (2.2, 5.1) |
| **9-11 months** | | | | | | | | |
| Energy (kcal) | **686** | **307** | 156 (93, 258) | 247 (168, 387) | 649 (432, 723) | 204 (129, 334) | 171 (102, 333) | 320 (258, 791) |
| Energy from fat (%) [c] | **30%** | **5%** | - | 18% | 27% | - | 12% | 19% |
| Proteins (g) | **9.1** | **3.1** | 2.3 (1.4, 3.8) | 7.7 (4.3, 13.3) | 22.0 (11.8, 26.5) | 3.0 (1.9, 4.9) | 4.3 (1.6, 9.4) | 8.0 (5.5, 22.3) |
| Vitamin A (µg RE) | **500** | **92** | 136 (81, 225) | 74 (39, 158) | 209 (149, 339) | 178 (113, 291) | 22 (6, 53) | 57 (13, 187) |
| Iron (mg) | **11** | **9.1** | 0.07 (0.04, 0.1) | 2.2 (1.3, 3.9) | 4.0 (3.5, 6.8) | 0.09 (0.05, 0.14) | 1.0 (0.5, 1.9) | 1.1 (0.7, 7.3) |
| Zinc (mg) | **3** | **2.3** | 0.4 (0.2, 0.6) | 1.0 (0.5, 1.7) | 2.8 (1.5, 3.5) | 0.5 (0.3, 0.8) | 0.6 (0.3, 1.3) | 1.2 (0.7, 3.0) |
| **12-23 months** | | | | | | | | |
| Energy (kcal) | **894** | **548** | 86 (43, 170) | 446 (297, 642) | 670 (512, 864) | 129 (65, 194) | 263 (154, 395) | 515 (337, 680) |
| Energy from fat (%) [c] | **30%** | **17%** | - | 21% | 25% | - | 13% | 17% |
| Proteins (g) | **10.9** | **5** | 1.3 (0.6, 2.5) | 14.6 (8.7, 23.5) | 23.2 (16.8, 32.2) | 1.9 (1.0, 2.9) | 5.8 (2.8, 10.9) | 14.2 (7.4, 21.5) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Vitamin A (µg RE) | **300** | **126** | 75 (38, 148) | 122 (68, 192) | 215 (131, 348) | 113 (56, 169) | 36 (13, 86) | 90 (33, 175) |
| Iron (mg) | **7** | **5.8** | 0.04 (0.02, 0.07) | 3.0 (2.0, 5.0) | 5.3 (3.3, 8.1) | 0.05 (0.03, 0.08) | 1.8 (0.9, 2.8) | 3.5 (2.2, 5.2) |
| Zinc (mg) | **3** | **2.4** | 0.2 (0.1, 0.4) | 1.8 (1.0, 2.7) | 2.9 (2.0, 3.9) | 0.3 (0.2, 0.5) | 0.9 (0.5, 1.4) | 2.0 (1.2, 2.8) |

[a] DRI (total), dietary reference intake for breastfed and non-breastfed children. Sources: energy (WHO/PAHO, 2003; WHO, 2005), protein (WHO, 1998), micronutrients (Dewey and Brown, 2003).

[b] DRI (CF), dietary reference intake for needs from solid/semi-solid foods for breastfed children with an average breast milk intake. Sources: energy and micronutrients for children aged 6-8 and 9-11 months (Dewey *et al.*, 2006), micronutrients for children aged 12-23 months (WHO, 1998), protein for children aged 6-8, 9-11 and 12-23 months (WHO, 1998).

[c] Mean percentage of energy from complementary food that should be provided as fat assuming an average breast milk intake (WHO/PAHO, 2003).

The analysis of the differences in average energy and nutrient intake between the urban and rural setting is presented in Table 3.4. The socio-demographic characteristics included in the analytical models were the same covariates used for the analysis with feeding indicators. When adjusting for these covariates, it was noticed that the urban-rural difference in energy intake from breast milk increased in 12%. In contrast, when considering the intake of complementary foods, those covariates explained partially the urban-rural difference. The adjusted analysis resulted in a reduction of 52% in setting difference of the total energy intake at complementary feeding stage. Furthermore, considerable reductions of the urban-rural difference in macronutrient (27-61%) and micronutrient (21-54%) intakes were observed. The impact of adjusting the analysis for differences in socio-demographic characteristics was more pronounced when excluding breast milk from the analysis. For that case it was observed that the urban-rural difference in average energy intake from solid/semi-solid foods was reduced in 66%, in the average macronutrient intakes in 51-81% and in the average micronutrient intakes in 50-71%. The urban-rural difference in energy intake from breast milk was 30% higher when the analysis was adjusted for maternal education only (data not shown) and 31% lower when the analysis was adjusted for facility-based delivery. The urban-rural difference in energy intake at complementary feeding was 38% lower when adjusting the analysis for facility-based delivery and 41% lower when adjusting for maternal education. Similarly, the urban-rural difference in energy intake from solid/semi-solid foods was mostly explained for the same two covariates, both resulting in a reduction of 30%.

After adjustment, not all covariates were significantly associated with the outcomes of the models. A higher average energy intake from breast milk was positively associated with a larger household size (33.4 kcal; 95% CI: 4.01, 62.8; $P$=0.026). The average total energy intake at complementary feeding stage was positively associated with facility-based delivery (56.3 kcal; 95% CI: 5.65, 107; $P$=0.030) and mothers working outside home (57.1 kcal; 95% CI: 20.2, 94; $P$=0.003). The average energy intake from solid/semi-solid foods were also positively associated with facility-based delivery (114 kcal; 95% CI: 80.3, 148; $P$<0.001), mothers working outside home (31.0 kcal; 95% CI: 1.16, 60.9; $P$=0.042), larger household size (28.5 kcal; 95% CI: 1.87, 55.1; $P$=0.036) and higher maternal education level (10.8 kcal; 95% CI: 6.04, 15.6; $P$<0.001).

**Table 3.4.** Mean energy and nutrient intake, with and without the breast milk intake estimation, and analysis of the difference and adjusted difference between urban and rural area: children aged 0-23 months (n=998), Cuenca (urban area, n=650) and Nabon (rural area, n=348).

| | **Cuenca** | **Nabon** | **Difference** [a] | | **Adjusted difference** [b] | |
|---|---|---|---|---|---|---|
| | mean (SD) | mean (SD) | Estimate (95% CI) | *P* | Estimate (95% CI) | *P* |
| **Breast milk** [c] | (n=399) | (n=255) | (n=558) | | (n=558) | |
| Energy (kcal) | 223 (205) | 255 (259) | 34 (15; 53) | <0.001 | 38 (8; 68) | 0.013 |
| **Solid/semi-solid foods** [d] | (n=588) | (n=273) | (n=738) | | (n=738) | |
| Energy (kcal) | 449 (318) | 340 (268) | -155 (-178; -132) | <0.001 | -53 (-78; -27) | <0.001 |
| Carbohydrates (g) | 70.9 (50.2) | 61.5 (47.6) | -16.6 (-20.2; -13.0) | <0.001 | -3.1 (-7.3; 1.1) | 0.143 |
| Proteins (g) | 15.5 (12.6) | 9.8 (10.1) | -7.2 (-8.2; -6.2) | <0.001 | -2.9 (-4.0; -1.8) | <0.001 |
| Fat (g) | 12.4 (10.4) | 6.6 (8.2) | -7.0 (-7.9; -6.2) | <0.001 | -3.4 (-4.4; -2.4) | <0.001 |
| Vitamin A (µg RE) | 161 (161) | 88 (139) | -87 (-100; -73) | <0.001 | -31 (-48; -14) | <0.001 |
| Iron (mg) | 3.7 (3.1) | 2.3 (2.4) | -1.8 (-2.1; -1.6) | <0.001 | -0.9 (-1.2; -0.7) | <0.001 |
| Zinc (mg) | 1.9 (1.5) | 1.3 (1.2) | -0.7 (-0.8; -0.6) | <0.001 | -0.2 (-0.4; 0.1) | <0.001 |
| **Total (CF)** [e] | (n=337) | (n=180) | (n=440) | | (n=440) | |
| Energy (kcal) | 492 (240) | 436 (217) | -81 (-109; -53) | <0.001 | -39 (-76; -2) | 0.037 |
| Carbohydrates (g) | 69.3 (39.8) | 64.9 (35.4) | -9.6 (-14.1; -5.1) | <0.001 | -3.7 (-9.5; 2.1) | 0.209 |
| Proteins (g) | 12.8 (8.9) | 9.1 (6.0) | -5.1 (-6.2; -4.0) | <0.001 | -3.7 (-5.0; -2.3) | <0.001 |
| Fat (g) | 19.2 (9.9) | 16.4 (10.6) | -2.6 (-3.7; -1.5) | <0.001 | -1.2 (-2.6; 0.3) | 0.117 |
| Vitamin A (µg RE) | 265 (160) | 223 (140) | -35 (-53; -17) | 0.001 | -16 (-43; 10) | 0.217 |
| Iron (mg) | 2.6 (2.2) | 1.6 (1.3) | -1.4 (-1.6; -1.2) | <0.001 | -1.1 (-1.4; -0.8) | <0.001 |
| Zinc (mg) | 1.7 (1.0) | 1.3 (0.7) | -0.5 (-0.6; -0.3) | <0.001 | -0.3 (-0.5; 0.2) | <0.001 |

[a] From linear regression model adjusted for child's age and sex and adjusted for clustered sampling design for Cuenca.

[b] From linear regression model adjusted for adjusted for child's sex and age, facility-based delivery; health seeking behavior, maternal age (years), maternal age at first delivery (years), maternal education (years), maternal working status, number of children <5 years, maternal marital status and additional income from migration, and adjusted for clustered sampling design for Cuenca.

[c] Breast milk intake in exclusively & predominantly breastfed infants and complementary fed infants (654 observations).

[d] Intake of solid/semi-solid foods (excluding breast milk), pooled from children at complementary and weaning feeding stage (861 observations).

[e] Intake at complementary feeding stage which include complementary foods and breast milk (517 observations).

### 3.3.4. Dietary sources

In total, a list of 249 food items from the urban area and a list of 120 food items from the rural area were compiled from the dietary 24-hr. However, most of the intake (>90%) constituted of 31 and 23 food items in the urban and rural area, respectively. Most food items were used as ingredients of composite recipes. The average energy density of the recipes at complementary feeding stage in Cuenca (0.96±0.92 kcal $g^{-1}$) was higher than in Nabon (0.84±0.78 kcal $g^{-1}$) ($P$<0.001). While the average energy density of the recipes at weaning stage was similar between Cuenca (1.03±1.0 kcal $g^{-1}$) and Nabon (1.0±0.95 kcal $g^{-1}$) ($P$=0.096) (data not shown).

In both areas, cow milk was the most important dietary source of energy and most nutrients. In Cuenca, cow milk was commonly consumed as such, in milk-shakes, mixed with cocoa powder, diluted with coffee, as basic ingredient for gruels and also small quantities of cow milk are usually added to soups. In Nabon, children received cow milk as such or diluted with coffee or soup. In Cuenca, milk was the main source of energy (30%), protein (43%), fat (55%), vitamin A (50%), iron (28%) and zinc (42%); whereas the main carbohydrates source was banana (15%). Also in Nabon, milk was the most important source of energy (16%), protein (27%), fat (40%), vitamin A (42%) and zinc (23%), while the main carbohydrates source was rice (19%) and the main iron source was white wheat bread (24%).

The distribution in food groups of the average daily energy intake for both settings at three different age groups was compared in Figure 3.2. Although there are differences in energy contribution, the proportional intake by food groups was similar for both settings. Overall, the urban infant diet was mainly based on dairy products (29%, mainly cow milk), cereals and cereal products (20%, mainly white wheat bread), fruits and vegetables (11%, mainly banana and apple) and tubers (11%, mainly potatoes). The rural diet was characterized by the high consumption of cereals and cereal products (26%, mainly polished rice), tubers (22%, mainly potatoes), dairy products (18%, mainly cow milk) and fruits and vegetables (15%, mainly banana and apple). Of notice, the consumption of vitamin A-rich fruits and vegetables, legumes and infant foods was

very rare in both areas (<5%). It is also noteworthy that consumption of flesh foods was unusual in the rural area (5%, mainly chicken) compared to the higher and more varied consumption of flesh foods in the urban area (10%, mainly chicken and beef). Although the relatively low percentage of consumption of fats and fatty snacks in both areas (<6%, mainly vegetable oil and cookies), a larger variety of those foods was observed in the urban area.

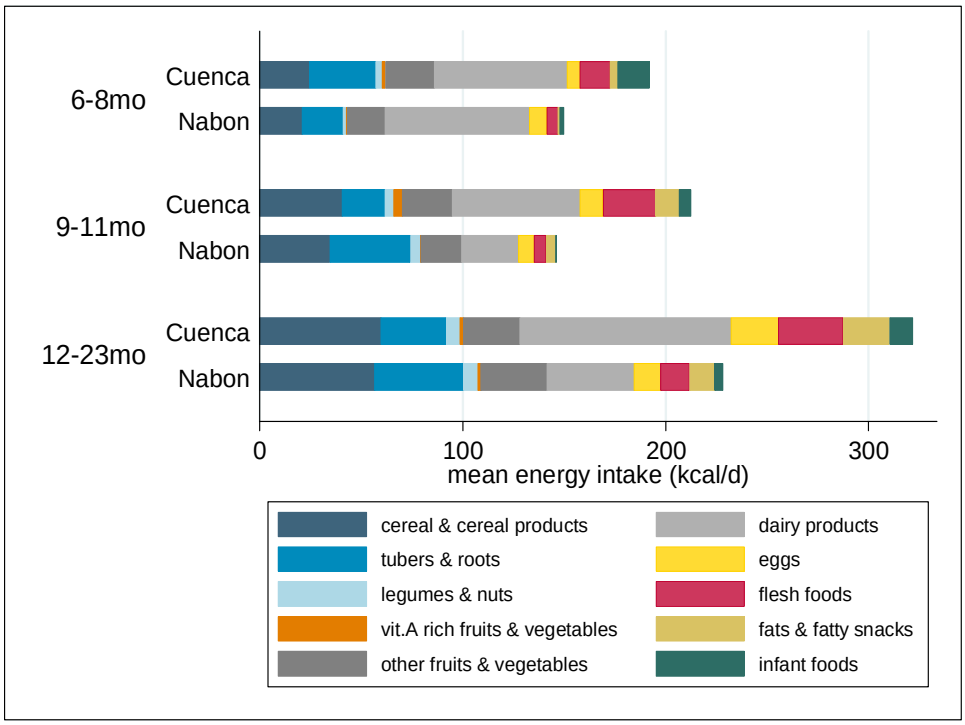


**Figure 3.2.** Average daily energy intake per food groups according to setting: children aged 6-23 months (n=752), Cuenca (urban area, n=497) and Nabon (rural area, n=255).

## 3.4. Discussion

The present results demonstrated differences in feeding patterns between rural and urban infants aged 0-23 months in the Ecuadorian highlands. Those differences were attributable to the quantity of food intake rather than the kind of basic diet. Although in the urban area the variety of foods consumed was much higher than in the rural area, the basic diet in both settings was similar and consisted of plant-based foods (cereal and cereal products, tubers, non-vitamin A fruits & vegetables) and dairy products, with cow milk being the most important source of energy and most nutrients. A rare consumption of vitamin A-rich fruits and vegetables in both areas was observed. It was also remarkable the limited consumption of flesh foods especially in the rural area. These findings are in line with a previous description of the child's diet in the Ecuadorian highlands that reported a higher consumption of filling but low nutrient dense foods and a lower consumption of animal-based food (Leonard *et al.*, 2000).

Breastfeeding practices were better in the rural area, whereas the nutrient and energy contribution from solid/semi-solid foods was considerably higher for urban infants. Nevertheless, those results might also suggest a likelihood of non-timely feeding, i.e. exclusive breastfeeding for longer than 6 months in Nabon and earlier introduction of solid/semi-solid foods in Cuenca.

In this study, the proportion of children with intake below the requirements was not established. For it, estimated average requirement values (EAR) are needed and those are not available for children under 2 years old (IOM, 2005). Instead, the referred DRIs corresponded to adequate intake (AI) or mean intake for healthy breastfed infants. The AIs do not provide sufficient evidence to establish the distribution of requirements. Therefore, only the prevalence of adequacy can be concluded when the average intake is above the AI value (Murphy and Poos, 2002; Otten *et al.*, 2006). In this context, the protein intake could be considered adequate for breastfed and non-breastfed children from the rural and urban area. It is noteworthy that in most cases the energy contribution from fat in solid/semi-solid foods for breastfed children was exceeded, being more pronounced in the urban area.

Poverty and socioeconomic inequity are important causes of poor dietary intake of young children (Darmon and Drewnowski, 2008; Salles-Costa *et al.*, 2010; Bhutta *et al.*, 2013b). In this study, it was hypothesized that individual socio-demographic characteristics could explain the actual rural-urban difference in food intake and feeding practices. Due to the large difference in SES between rural and urban households, the inclusion of the SES as a covariate for the evaluation of the aforementioned urban-rural difference was not possible. Maternal education and facility-based delivery were the most important covariates that explained the difference between the urban and rural setting. Both socio-demographic characteristics were positively associated in the urban ($P<0.001$) and rural area ($P<0.001$) (data not shown). In addition, both characteristics were considerably better in the urban area.

Regarding the intake of breast milk, it was observed that the urban-rural difference systematically remained. This suggested that, besides the considered socio-demographic characteristics, the higher consumption of breast milk in the rural area might be associated with a community effect, i.e. environmental particularities in the rural area that could promote breastfeeding such as family behaviors or cultural beliefs as described previously (Torgerson and Edwards, 2013). On the other hand, this pro-breastfeeding behavior could also be a result of previous governmental programs aimed at the rural poor dealing with child nutrition. The most important covariate that explained the urban-rural difference in breast milk intake was the fact that the child born at medical facilities. The better breastfeeding practices in the rural area did not seem to be related to this factor that by itself cannot directly influence on breastfeeding or food intake. Facility-based delivery might also be associated to pre- and/or post-partum nutritional counseling. Although facility-based delivery may also be associated with

better-off households, it could be the case in the urban area. However, about 90% of the children in the urban area were born at health facilities independently of their household SES (data not shown). In addition, it cannot be excluded that poverty and food scarcity could lead mothers to breastfeed more as previously suggested for indigenous women in Ecuador (World-Bank, 2007). Moreover, although less optimal breastfeeding practices in rural communities have been described (Kamudoni *et al.*, 2007; Senarath *et al.*, 2007), longer breastfeeding has been usually observed among the more rural countries of Latin America (Perez-Escamilla, 2003). Also, the positive association observed between a higher intake of breast milk with larger household size might have cultural bases or be driven by socioeconomic conditions.

As aforementioned, facility-based delivery together with maternal education were the most important covariates that explained the rural-urban difference in average energy intake. In the adjusted analysis, energy intake from solid/semi-solid foods was positively and significantly associated with facility-based delivery, as well as with maternal education, maternal work outside home and larger household size (with more children under 5 years old). The direct relation of facility-based delivery is unclear but might represent a consequence of health consciousness. Higher level of maternal education in the urban area might directly (child health consciousness) or indirectly (better-off household environment) favor child food intake. Maternal education was also positively associated with timely introduction of solid/semi-solid foods (6-8 months) and with the energy intake from solid/semi-solid foods. Nevertheless, a residual urban-rural difference remained. It was surprising to observe a positive association between a higher intake of solid/semi-solid foods with larger household size in the urban area, while the opposite might be expected. These results support the described complexity of urbanization which has been characterized by higher levels of education; more employment opportunities outside home for both men and women, greater necessity of income generation to cover basic needs, and different family structure and social networks which may affect the availability of childcare alternatives. Furthermore, as a result of urbanization, females could become the head of the household turning into difficulties regarding childcare and income generation (Ruel, 2000). On the other hand, scarce information is available about the lifestyle in rural areas and how socio-demographic characteristic interact.

Although vast information about predictors of good feeding practices at early childhood is available, data on rural-urban comparisons is very limited. The found significant associations between socio-demographic determinants and feeding practices were also observed in other studies in urban areas of developing countries. For instance, the association of facility-based delivery with exclusive breastfeeding (Aidam *et al.*, 2005), multi-parity with early breastfeeding (Radwan, 2013) and maternal education with timely introduction of complementary foods (Kudlova and Rames, 2007) have been described. On the other hand, the majority of the studies on child food intake had special focus on diet adequacy or dietary diversity, and few studies have addressed the

impact of socio-demographic factors on the overall child food intake. In particular, factors such as low parental education, low equivalent income and food insecurity index have been associated with different patterns on dietary diversity (Sausenthaler *et al.*, 2007; Salles-Costa *et al.*, 2010).

A number of limitations of this study need to be addressed. First, the cross-sectional design of the study allows describing only associations at population level and non-causal relationships could be established. Second, the study was questionnaire-based and some questions that required a good memory were vulnerable to recall bias such as breastfeeding practices and dietary recalls. Third, breast milk intake was based on a proxy estimation using a general conversion factor. Recall bias is expected because this approach relied on maternal recall which could also differ between settings. Fourth, possible bias related to the use of different food composition databases can arise. For instance, it was observed that white wheat bread was the major source of iron in the rural area which suggested that the actual source was fortified flour. In fact, data on the composition database corresponded to fortified white wheat bread as it is usually produced in the urban area. Unfortunately detailed information about fortification was unknown by the respondents. Finally, the described feeding patterns for the rural area were based on a single 24-hr and therefore day-to-day variation was misreported. However, it must be considered that information was collected in almost the total children population size of the rural area (400 infants).

## 3.5. Conclusions

This part of the study was focused on the urban-rural differences in feeding patterns and food intake of children from birth up to 23 months of age in the Ecuadorian highlands. In both settings, the basic diet was characterized by a high consumption of plant-based foods. Better breastfeeding practices and higher intake of breast milk were observed in the rural area. In contrast, higher intake of solid/semi-solid foods was observed in the urban area. The living conditions that could offer advantaging environments for better breastfeeding practices in the rural areas, such as community networks, should be further studied. Maternal education and facility-based delivery were the most important socio-demographic characteristic that explained the urban-rural differences in food intake and feeding indicators. Both, higher maternal education and facility-based delivery were defining characteristics in the urban area. It is hypothesized that those factors could be directly associated with health consciousness towards appropriate feeding practices. A potential indirect association with better-off households is also suggested. Therefore, efforts to improve the access to health services and education in the rural area could substantially support child feeding practices in this region.

# CHAPTER 4

## Mycotoxin co-occurrence in rice, oat flakes and wheat noodles used as staple foods in Ecuador

*Redrafted after:*

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# 4. Mycotoxin co-occurrence in rice, oat flakes and wheat noodles used as staple foods in Ecuador

**Abstract**

In this chapter, the co-occurrence of aflatoxin $B_1$ ($AFB_1$), $B_2$ ($AFB_2$), $G_1$ ($AFG_1$) and $G_2$ ($AFG_2$), ochratoxin A (OTA), deoxynivalenol (DON), fumonisin $B_1$ ($FB_1$), zearalenone (ZEN), and HT-2 and T-2 toxin in the main Ecuadorian staple cereals (rice, oat flakes and yellow and white wheat noodles) was evaluated. An ultra high performance liquid chromatography/time of flight mass spectrometry (UHPLC/TOFMS) method was developed and validated to screen for the presence of these mycotoxins in those cereal matrices. Matrix-matched calibration curves were used to compensate ion suppression and extraction losses and the recovery values were in agreement with the minimum requirements of the regulation 401/2006/EC (70-110%). For most mycotoxins, the LOD's obtained allowed detection in compliance with the maximum permitted levels set in the regulation EC/2006/1881, with exception of OTA in all cereals and $AFB_1$ in yellow noodles. Additional analyses of OTA in oat flakes and wheat noodles were performed by HPLC with fluorescence detection. High rates of contamination were observed in paddy rice (23% DON, 23% $FB_1$, 7% $AFB_1$, 2% $AFG_1$ and 2% $AFG_2$), white wheat noodles (33% DON, 5% OTA) and oat flakes (17% DON, 2% OTA and 2% $AFB_1$), whereas the rates of contamination were lower in polished rice (2% $AFG_1$ and 4% HT-2 toxin) and yellow noodles (5% DON). Low rates of co-occurrence of several mycotoxins were observed only for white wheat noodles (5%) and paddy rice (7%). White noodles were contaminated with DON and/or OTA, while combinations of $AFG_1$, $AFB_1$, DON and $FB_1$ were found in paddy rice. Yellow noodles were contaminated with DON only; oat flakes contained DON, OTA or $AFB_1$, and polished rice was contaminated with $AFG_1$ and HT-2 toxin.

## 4.1. Introduction

Mycotoxins are toxic secondary metabolites produced by different fungal species, growing under a wide range of climatic conditions on agricultural commodities. Contamination may occur throughout the food chain and is considered as a serious worldwide safety problem for the whole agri-food chain (FAO/IAEA, 2001; Shephard, 2008a; Bhat *et al.*, 2010). Hundreds of mycotoxins have been discovered; however, a limited number are significantly threatening food safety. Based on the adverse implications on human health and agricultural productivity, the most important mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, HT-2 and T-2 toxins (FAO/IAEA, 2001; Shephard, 2008a; Bhat *et al.*, 2010).

Fungal growth is mainly triggered by high water activities (0.80-0.99) and warm temperatures (25-30°C) and often leads to the co-occurrence of several mycotoxins in foodstuffs that could induce synergistic toxicological effects (FAO/IAEA, 2001; Desmarchelier *et al.*, 2010). Nowadays, several multimycotoxin methods based on LC-MS techniques are being developed to enable sensitive, reliable and fast identification and quantification of mycotoxins (Shephard *et al.*, 2013). LC/TOFMS is a valuable technique for multimycotoxin analysis (Tanaka *et al.*, 2006; Senyuva *et al.*, 2008; Zachariasova *et al.*, 2010) with broad detection capabilities that enables retrospective data treatment of non-target compounds (Ojanpera *et al.*, 2006).

Ecuador, a country located at the northwest of South America, could provide favorable conditions for fungi development and mycotoxin production (Pacin *et al.*, 2002). This unique climate diversity is caused by the presence of the Andes mountain range, the sea influence and the Equator location. Very limited published data are available about co-occurrence of mycotoxins in staple foods in South American countries in general and in Ecuador particularly (FAO, 2004; Sabino, 2011). Low occurrences of OTA, DON, T-2 toxin and aflatoxins in Ecuadorian paddy rice and polished broken rice fractions had been formerly reported (Mühlemann *et al.*, 1997a, b), but those products are not intended for human consumption. The main energetic source of the Ecuadorian diet is cereals-based (35%) (FAO, 2001; Bermudez and Tucker, 2003), and staple cereals such as rice, wheat products and oats are known to be prone to mycotoxin contamination (FAO/IAEA, 2001; Shephard, 2008a; Yazar and Omurtag, 2008). Ecuador is the major consumer of rice amongst the Andean countries (119.2 kg/capita/year) and the third most representative producer as part of the CAN (Nations of the Andean Communities) (INEC, 2011a). On the other hand, wheat is one of the most consumed cereals in Ecuador and the national production is not self-sufficient. About 98% of the wheat consumed is mainly imported from Canada and Unites States (INEC, 2011b; USDA, 2013a). Similar trends of consumption and production are observed for oat, but it is also imported from Chile (FAO, 2001).

This chapter presents the part of the study which aimed at reporting the occurrence of

mycotoxins in Ecuadorian staple foods, i.e. polished rice, oat flakes and wheat noodles (white and yellow). The occurrence of mycotoxins in paddy rice is also reported as an indicator of pre-milling contamination. The major mycotoxins of health concern $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, OTA, DON, $FB_1$, ZEN, HT-2 and T-2 toxin were screened by an optimized UHPLC/TOFMS method. Additional analyses of OTA in oat flakes and wheat noodles using HPLC-FLD were performed. This mycotoxin evaluation in staple cereals and cereal-based products allowed prioritizing the major mycotoxins present in those commodities to support further risk management options.

## 4.2. Methodology

### 4.2.1. Chemicals and reagents

LC-MS grade water, acetonitrile, methanol, acetic acid; formic acid, ammonium acetate, sodium hydroxide and isopropanol were supplied by Fluka (Steinheim, Germany). Ochraprep immunoaffinity columns were purchased from R-Biopharm Rhône (Glasgow, UK). Standards, as solid pure extracts, of $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, OTA, DON, $FB_1$, HT-2 toxin, T-2 toxin, ZEN and zearalanone (ZAN), as well as phosphate-buffered saline (PBS) solution were supplied by Sigma–Aldrich (St. Louis, MO, USA).

The standards were reconstituted with acetonitrile, except $FB_1$ for which acetonitrile/water 1:1, v/v was used. Aliquots of standard solutions were dried under a gentle stream of nitrogen and kept at 4°C, except ZEN, ZAN, HT-2 and T-2 which were kept at -20°C. For MS calibration, a sodium acetate solution was prepared by mixing 0.1% acetic acid and 1% 1M NaOH in water/isopropanol mixture (1:1).

### 4.2.2. Standard solutions

For validation experiments for the multimycotoxin method by UHPLC/TOFMS, stock solutions of 1 µg $mL^{-1}$ were prepared by reconstitution of the dried standards with methanol. A mixture of methanol/water (1:1, v/v) containing 5 mM of ammonium acetate (pH 8.4) was used for further dilutions. A multi-standard stock solution was freshly prepared by mixing suitable amounts of individual standards solutions to cover a concentration level roughly the maximum permitted limits in cereals set by the European Commission in the Regulation EC/1881/2006 (European-Commission, 2006b, 2012). For HT-2 and T-2 toxin, no maximum levels were available at the time of these experiments. Therefore the concentration used to prepare the multi-standard stock solution were the same as for other mycotoxins which showed comparable signal intensities at similar concentrations.

For validation experiments for OTA analyzed by HPLC-FLD, a stock solution of 1 µg $mL^{-1}$ was prepared by reconstitution of the dried standard with acetonitrile. Further dilutions were freshly prepared with a mixture of acetonitrile /water (1:1, v/v).

### 4.2.3. Sampling and sampling frame

Two sampling plans (bulk and retail) were designed in accordance with the European Commission Regulation 401/2006/EC (European-Commission, 2006a) and complemented with relevant information from Codex Alimentarius (Codex-Alimentarius, 2008).

Rice samples were collected from the biggest rice mills of the country (called first category mills) located at the lowlands of the coastal provinces of Guayas and Los Ríos, which are the main rice-producing zones in Ecuador (98.7% of the national production) (INEC, 2011a). Both provinces are situated at the Pacific coast and have an average temperature of 25°C. Los Ríos province is located at approximately 6-11 meters above sea level and it is submitted to tropical monsoons varying from dry and cool season from June to December to hot and humid conditions from December to June. Guayas province is located at approximately 4-6 meters above sea level and its weather ranges from a tropical savannah at the north to a tropical monsoon in the southern regions. The growth and harvest of the collected rice samples occurred during the rainy season. Incremental samples were collected from different sections of a bulk lot, i.e. 10 times 1 kg, and the gathered aggregate sample of 10 kg was mixed thoroughly. A final amount of 1 kg was taken as laboratory sample and the remainder was sent back to the rice mill (paddy rice) or primary storage place (polished rice). In total, 62 mills in Los Rios and 61 mills in Guayas were visited and those compiled the rice production of 18 cantons in Guayas and 12 cantons in Los Ríos. A total of 121 samples of paddy rice (60 from Los Ríos and 61 from Guayas) and 125 samples of polished rice (64 from Los Ríos and 61 from Guayas) were gathered from May to July 2010. Both types of samples were collected during the same visit; therefore those did not belong to the same lot before and after milling. Upon collection, samples of paddy rice were dried using an air oven set at 50°C for 24 hours to prevent fungal infestation. In addition, the supervisor of each mill was surveyed during sampling. The survey comprised questions regarding use of fertilizers and pesticides in the field, moisture content of the incoming and polished rice, impurities of the incoming rice, storage time before and after milling process and management of mold infestation.

Samples of oat flakes, yellow-alkaline and white wheat noodles (Fiocchetti type) were bought at retail in open markets and supermarkets in Cuenca (urban) and Nabon (rural) cantons of Azuay province, in the southern Ecuadorian highlands. Cuenca is located at approximately 2,550 meters above sea level. Nabon is located at 3,000 meters above sea level and at 70 km from Cuenca. Incremental samples were either the smallest packages available until completing 1 kg (2-5 packages) or enough amounts bought by weight from big sacks. At laboratory level, the content of those packages was thoroughly homogenized. A total of 70 samples of oat flakes, 63 samples of white wheat noodles and 65 samples of yellow wheat noodles were collected during February-March 2010.

Upon collection, samples were kept in dark polyethylene bags at room temperature and finally ground just before analysis. Half of the samples were randomly selected and shipped to Belgium for multimycotoxin analysis.

### 4.2.4. Sample preparation

#### 4.2.4.1. Multimycotoxin analysis by UHPLC/TOFMS

An amount of 0.5 grams of homogenized ground sample was extracted with 2 mL of the solvent mixture acetonitrile/water/acetic acid, 79:20:1 (v/v/v) and the suspension was shaken using a vortex. The mixture was further mixed on a rotary shaker (Labinco, Breda, The Netherlands) for 90 min and then centrifuged for 2 min at 4053 g (Sigma 4k15. Buckingham, UK). A volume of 750 µL of extract was transferred into microtubes and dried under a gentle stream of nitrogen. The dried extract was redissolved in 1 mL methanol and frozen for one hour at -24°C. This methanolic extract was subsequently removed and the walls of the microtube were rapidly washed with another 0.5 mL of ice-cold methanol. The combined extract was dried under nitrogen and finally reconstituted in 750 µL of mobile phase A. After vortexing and sonication, the sample was filtered (0.2µm filter) and a volume of 20 µL was injected.

#### 4.2.4.2. OTA analysis by HPLC

Twenty-five (25) grams of finely milled sample were extracted with 100 mL of the solvent mixture acetonitrile/water, 60:40 (v/v). The suspension was shaken in a horizontal shaker at 300 rpm for 30 min. The mixture was centrifuged for 10 min at 2403 g (Hettich EBA 20. Tuttlingen, Germany). A volume of 2 mL of extract was diluted with 22 mL of PBS solution. After vigorous shaking, the solution was applied to immunoaffinity clean-up columns (Ochraprep, R-Biopharm Rhône), which were previously brought to room temperature and conditioned with 3 mL of PBS solution. The diluted extract (24 mL, equivalent to 0.5 g of sample) was passed at a flow rate of 2-3 mL $min^{-1}$. The column was washed with 10 mL of PBS solution followed by 10 mL of water at a flow rate of 5-6 mL $min^{-1}$. The column was dried by applying vacuum for 10 seconds. OTA was eluted from the column with 1.5 mL of the mixture methanol/acetic acid, 98:2 (v/v) passing through by gravity. The eluate was back-flushed twice using a syringe and then air was pushed through the column. The collected eluate was finally diluted with 1.5 mL of water, which also passed through the column at a flow rate of 2-3 mL $min^{-1}$. The sample was filtered (0.45µm filter) and a volume of 100 µL was injected.

### 4.2.5. Instrumental parameters

#### 4.2.5.1. UHPLC/TOFMS conditions for multimycotoxin analysis

UHPLC separation was achieved on an UltiMate 3000 RSLC system (Dionex, Breda,

The Netherlands), composed of a vacuum degasser, binary pump, cooled autosampler, column oven (30°C), and equipped with a Zorbax Eclipse XDB C18 column (1.8 µm, 2.1 x 100 mm, Agilent Technologies, Waldbronn, Germany). Mobile phase A consisted of water/methanol/acetic acid 94:5:1 (v/v/v) and mobile phase B of methanol/water /acetic acid 97:2:1 (v/v/v), both containing 5 mM of ammonium acetate with pH 3.25 (eluent A) and pH 5.1 (eluent B). The gradient was: 0-14 min linear increase from 30 to 95% B, 14-14.1 min linear increase to 100% B and hold for 3.9 min, followed by re-equilibration of the column for 5 min, all applying a flow rate of 0.2 mL $min^{-1}$. The RSLC system contained a splitless interface to a time-of-flight mass spectrometer (micrOTOF II, Bruker Daltonics, Bremen, Germany) with a resolving power of 16,500-18,000 FWHM. It was equipped with an orthogonal electrospray ionization source (ESI) operating in positive mode, using a mass range of 50–1,000 Da for m/z acquisition. The MS method contained 4 time segments: 0-0.5 min for calibration, 0.5-11.7 min for detection of DON, $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$ and HT-2 toxin, 11.7-12.35 min for the detection of $FB_1$, and 12.35-14.1 min for detection of T-2 toxin, OTA and ZEN. MS parameters, common for all segments, were capillary voltage 6,000 V, nebulizer pressure 2 bars, dry gas temperature 200°C and dry gas flow 7 L $min^{-1}$. The applied capillary exit voltage was 90 V, skimmer 1 voltage 30 V and hexapole RF 250 for segments 1, 2 and 4. For segment 3 ($FB_1$), those settings were 105 V, 35 V and 600 V respectively. At the beginning of every run, the MS was calibrated with a sodium acetate calibrant solution.

Quantification was performed using matrix-matched calibration curves (MMCC), which were constructed by spiking the multi-standard solution into testing matrices before extraction at 3 concentration levels corresponding to 0.5-, 1- and 1.5-times an individual concentration of each mycotoxin: 640 µg $kg^{-1}$ for DON; 8 µg $kg^{-1}$ for $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$; 80 µg $kg^{-1}$ for HT-2 toxin and ZEN, 40 µg $kg^{-1}$ for T-2 toxin and OTA, and 200 µg $kg^{-1}$ for $FB_1$. The MMCC used to calculate the limit of detection (*detailed in section 4.2.6.1*) were also used for quantification purposes. For internal quality control purposes, a fixed concentration of 80 µg $kg^{-1}$ of ZAN was added. After spiking, the samples were kept overnight at room temperature and protected from light to allow the equilibration of the multi-standard working solution with the cereal matrix.

#### 4.2.5.2. HPLC conditions for OTA analysis

OTA in oat flakes and wheat noodles was analyzed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of an isocratic pump, vacuum degasser, autosampler, column oven (25°C), and equipped with a Zorbax Eclipse C18 column (5µm, 4.6 x 250 mm, Agilent Technologies, USA). An isocratic elution was applied with a mobile phase containing a mixture of acetonitrile/water/acetic acid 50:49:1 (v/v/v) at a flow rate of 1 mL $min^{-1}$. Fluorescence detection was carried out at 247 and 480 nm of excitation and emission wavelengths, respectively. Quantification of OTA was performed by measurement of the peak areas at the OTA retention time and

comparing them with a twelve-point calibration curve (0.1–250 ng $mL^{-1}$, $R^2$ = 0.9996). The resultant OTA concentration was finally corrected by the corresponding recovery.

### 4.2.6. Method performance

For validation experiments, samples of polished rice, wheat noodles and oat flakes taken from the batches of real samples were used as testing matrices to decrease as much as possible the matrix variability in the method performance. Traces of contamination were corrected using the standard addition technique.

#### 4.2.6.1. Multimycotoxin analysis by UHPLC/TOFMS

To assess linearity, calibration curves of multi-standard solutions in pure solvent were plotted at 12 concentration levels: 1.0, 3.0, 5.0, 10, 20, 30, 50, 75, 100, 160, 200 and 240 ng $mL^{-1}$. In addition, the linearity of the MMCC was assessed. The limits of detection (LOD) and quantification (LOQ) were assessed by spiking testing samples before extraction at 6 concentration levels (8, 20, 40, 100, 300 and 400 µg $kg^{-1}$ of DON, and 2, 4, 8, 20, 40 and 80 µg $kg^{-1}$ for the other mycotoxins) in duplicate. The limit of detection was determined using the equation **LOD** = 3 $s_{bl}$/S, in which $s_{bl}$ is the standard deviation of the intercept and S is the slope of the respective linear regression calibration curve. The limit of quantification was calculated by **LOQ** = 6 $s_{bl}$/S (Taverniers *et al.*, 2004). Recoveries were calculated according to (Desmarchelier *et al.*, 2010) and were determined based on six replicates MMCC constructed for each matrix at 3 concentration levels (detailed in section *4.2.5.1.*). The intra-day precision was assessed based on the replicates of the recovery experiments, while inter-day precision was performed by spiking one testing sample before extraction at 1-fold concentration of the multi-standard solution during 3 consecutive days. The matrix effects were assessed determining the signal suppression-enhancement (SSE) calculated according to (Sulyok *et al.*, 2006) from the comparison of the slope of the calibration curve of extracts spiked just before injection and the slope of the calibration curve of the standard working solutions. The SSE experiments were performed in duplicate at 4 concentration levels: 25, 50, 75 and 100 µg $kg^{-1}$ for all mycotoxins.

#### 4.2.6.2. OTA analysis by HPLC

Linearity was assessed by plotting calibration curves of the standard in pure solvent at 12 concentration levels of the standard (0.1, 0.5, 1.0, 2.0, 5.0, 10, 25, 50, 75, 100, 150, 175 and 250 ng $mL^{-1}$). LOD's, LOQ's and recovery experiments were performed in duplicate at 6 concentration levels (1.5, 6, 12, 30, 60, 120 µg $kg^{-1}$). The intra-day precision was assessed by spiking before extraction of a testing sample at 30 µg $kg^{-1}$ of OTA in triplicate; while inter-day precision was performed by spiking a testing sample at the same concentration during 3 consecutive days.

### 4.2.7. Data evaluation

UHPLC/TOFMS data analysis was performed using the software DataAnalysis version 4.0 SP 2. TargetAnalysis$^{TM}$ software (Bruker Daltonics, Bremen, Germany) was used for generation of extracted ion chromatograms (EIC) of the acquired $[M+H]^+$ ions from the total ion chromatograms (TIC). Identification and distinction between true- and false-positive results was based on retention time deviation, mass accuracy and SigmaFit$^{TM}$ algorithm which is a rate for the agreement of the theoretical and measured isotopic patterns (Ojanpera *et al.*, 2006). For all compounds, threshold parameters of detection were: mass accuracy of 5 ppm, and m/z tolerance of 5 ppm, extraction mass window of 15 mDa, mSigma of 50 and retention time window of 0.15 min. Chemstation 3D software (Agilent 1200) was used to control the HPLC system and for single data processing. Descriptive analyses of rice agricultural practices and comparison tests (ANOVA) were performed in Stata 10.0 (Stata Corporation, College Station, Texas, USA). Contamination rates for each mycotoxin and staple cereal was determined together with the standard deviation of a sample proportion ($SD_p$) calculated according to (Uyttendaele *et al.*, 2009) as $\mathbf{SD_p} = 100 \times \sqrt{p(1-p)/n}$, in which p is the proportion of positive samples and n is the total number of analyzed samples.

## 4.3. Results & Discussion

### 4.3.1. UHPLC/TOFMS method performance

The MS parameters were tuned for each mycotoxin by direct infusion of individual standard solutions at a concentration of 1 µg $mL^{-1}$. Specific MS settings (capillary exit voltage, skimmer 1 voltage and hexapole RF) were necessary for $FB_1$ because of its higher molecular mass, compared to the other mycotoxins. Positive and negative electrospray conditions were evaluated and all mycotoxins were best detected in the positive mode, as $[M+H]^+$ ions for aflatoxins, DON, $FB_1$, OTA, ZEN and ZAN. HT-2 and T-2 toxins were detected as $[M+NH4]^+$ ions. The less abundant ions were also detected and used as qualifiers (Table 4.1). The mobile phases were selected based on previous multimycotoxin analytical methods (Sulyok *et al.*, 2006). Both phases were acidified with 1% of acetic acid to improve the peak shape and ionization (Sulyok *et al.*, 2006; Desmarchelier *et al.*, 2010). A low flow rate was applied in order to change the MS settings along the run and to reduce matrix effects due to co-elution (Songsermsakul and Razzazi-Fazeli, 2008). Several chromatographic gradients were evaluated to achieve good peak shapes and separation. A better peak intensity was however attained for DON using a different gradient, but this affected the separation of the other mycotoxins eluting later in the chromatogram. Consequently, the applied gradient was considered as the best compromise for a good separation of all mycotoxins, which was established within 14 min. (Figure 4.1).

**Table 4.1.** Overview of detected ions, molecular formula, theoretical mass/charge ratio (m/z), average retention times (RT) and SigmaFit™ (*mSigma*) values for the most abundant ion of each mycotoxin.

| Mycotoxin | Ion $[M+H]^+$ / exact m/z | Ion $[M+Na]^+$ / exact m/z | Ion $[M+NH_4]^+$ / exact m/z | RT (min) | mSigma |
|---|---|---|---|---|---|
| DON | $C_{15}H_{20}O_6$ [a]/ 297.133265 | $C_{15}H_{19}O_6Na$ / 319.115209 | | 2.3 | 13 |
| $AFG_2$ | $C_{17}H_{14}O_7$ [a]/ 331.081229 | $C_{17}H_{13}O_7Na$ / 353.063174 | | 6.8 | 8 |
| $AFG_1$ | $C_{17}H_{12}O_7$ [a]/ 329.065579 | $C_{17}H_{11}O_7Na$ / 351.047524 | | 7.6 | 8 |
| $AFB_2$ | $C_{17}H_{14}O_6$ [a]/ 315.086315 | $C_{17}H_{13}O_6Na$ / 337.068259 | | 8.3 | 8 |
| $AFB_1$ | $C_{17}H_{12}O_6$ [a]/ 313.070665 | $C_{17}H_{11}O_6Na$ / 335.052609 | | 9.0 | 6 |
| HT-2 | $C_{22}H_{32}O_8$ / 425.216994 | | $C_{22}H_{35}O_8N$ [a]/ 442.243544 | 11.3 | 11 |
| $FB_1$ | $C_{34}H_{59}NO_{15}$ [a]/ 722.395747 | $C_{34}H_{58}NO_{15}Na$ / 744.377691 | | 12.0 | 8 |
| T-2 | $C_{24}H_{34}O_9$ / 467.227559 | | $C_{24}H_{37}O_9N$ [a]/ 484.254108 | 12.5 | 9 |
| OTA | $C_{20}H_{18}NO_6Cl$ [a]/ 404.089541 | $C_{20}H_{17}NO_6ClNa$ / 426.071486 | | 13.4 | 12 |
| ZEN | $C_{18}H_{22}O_5$ [a]/ 319.154000 | $C_{18}H_{21}O_5Na$ / 341.135945 | | 13.7 | 9 |

[a] Most abundant ions for all matrices.

The method performance was assessed in terms of linearity, limits of detection and quantification, recovery, signal suppression-enhancement, inter- and intra-day precision. Good linearity for all mycotoxins was achieved, both in the multi-standard solutions ($R^2 > 0.99$) and in the MMCC ($R^2 > 0.98$). The obtained values of LOD and LOQ are presented in Table 4.2. Most of the LOD's were similar to previous studies in cereals, in which crude extracts were analyzed (Sulyok *et al.*, 2006; Zachariasova *et al.*, 2010). Worldwide, several regulations for maximum levels of mycotoxins in foodstuffs intended for human consumption have been harmonized between countries belonging to some economic/trading communities (FAO, 2004; Van Egmond *et al.*, 2007). Among all, the European regulations consider the largest amount of mycotoxins in several commodities, and have adopted the lowest permitted levels for human consumption (FAO, 2004). Since no regulation is enforced in Ecuador, the obtained LOD's and LOQ's were compared with the maximum permitted levels established by the European commission (European-Commission, 2006b, 2012, 2013). The LOD's of DON were the highest among all matrices; however, those were low enough for detection in

compliance with the European regulation. Similar compliance was achieved for $AFB_1$ in oat, white noodles and paddy rice, and ZEN in polished rice. The LOD's of OTA in all cereals and $AFB_1$ in yellow noodles were higher than the set levels in the mentioned regulation. Whereas, the LOD's and LOQ's of $FB_1$ in all cereals; $AFB_1$ in polished rice, and ZEN in all cereal matrices except polished rice were below those set limits.

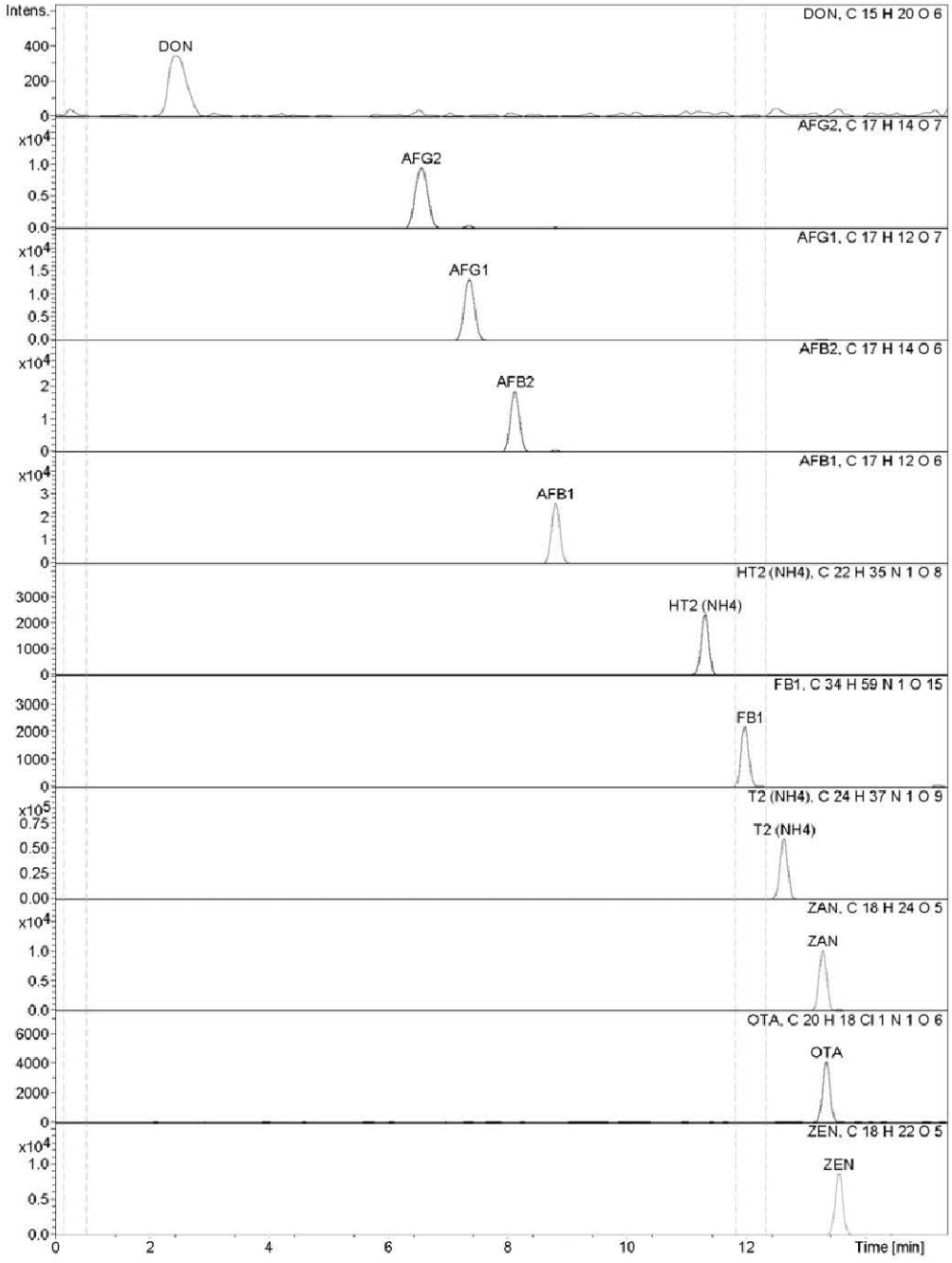



**Figure 4.1.** UHPLC/TOFMS chromatograms obtained from a blank sample of milled rice spiked at 80 μg $kg^{-1}$ of all mycotoxins.

**Table 4.2.** LOD's and LOQ's for polished and paddy rice, oat flakes, white wheat noodles (WWN) and yellow wheat noodles (YWN) and maximum levels of contamination allowed in cereals for direct human consumption, all expressed in µg $kg^{-1}$.

| Mycotoxin | Max. permitted levels in cereals | Polished rice | | Paddy rice | | Oat flakes | | WWN | | YWN | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LOD | LOQ | LOD | LOQ | LOD | LOQ | LOD | LOQ | LOD | LOQ |
| DON | 750 | 24 | 48 | 29 | 59 | 26 | 53 | 53 | 107 | 84 | 167 |
| $AFG_2$ | - | 1 | 3 | 3 | 5 | 2 | 4 | 6 | 11 | 9 | 18 |
| $AFG_1$ | - | 1 | 1 | 7 | 14 | 1 | 3 | 2 | 4 | 7 | 14 |
| $AFB_2$ | - | 2 | 3 | 4 | 9 | 1 | 3 | 4 | 8 | 6 | 12 |
| AFt | 4 (all cereals); 10 (rice) | - | - | - | - | - | - | - | - | - | - |
| $AFB_1$ | 2 (all cereals); 5 (rice) | 1 | 2 | 4 | 8 | 2 | 4 | 2 | 3 | 8 | 15 |
| $FB_1$ | 400 [a] | 16 | 32 | 10 | 19 | 8 | 16 | 15 | 30 | 11 | 22 |
| HT-2 | - | 20 | 41 | 7 | 15 | 12 | 24 | 5 | 9 | 16 | 31 |
| T-2 | - | 2 | 5 | 6 | 11 | 2 | 3 | 3 | 5 | 7 | 15 |
| Sum of HT-2 + T-2 | 50 (cereals)[b]; 25 (pasta); 200 (oat flakes) | - | - | - | - | - | - | - | - | - | - |
| OTA | 3 | 9 | 18 | 8 | 17 | 8 | 15 | 7 | 14 | 15 | 30 |
| ZEN | 75 | 39 | 77 | 22 | 45 | 3 | 7 | 27 | 54 | 23 | 46 |

AFt, total aflatoxins (sum of $AFG_2$+$AFG_1$+$AFB_2$+$AFB_1$)

[a] Sum of $FB_1$ + $FB_2$, only established for maize based foods

[b] Cereal grains for direct human consumption (different than oat and maize) that have undergone drying, cleaning, de-husking and sorting processes and on which no further cleaning and sorting processes will be performed before their further processing in the food chain

Variations in SSE of the different cereal matrices are presented in Table 4.3. The observed matrix effects were strongly influenced by the type of matrix and the nature of the analyte as observed in other studies (Songsermsakul and Razzazi-Fazeli, 2008; Frenich *et al.*, 2009). Thus, SSE differed significantly amongst different cereal matrices ($p<0.05$) and different mycotoxins ($p<0.05$). The matrix effect phenomenon is mainly caused by the presence of co-eluting matrix components in the crude extract, hampering the precision and sensitivity of the analysis (Antignac *et al.*, 2005; Songsermsakul and Razzazi-Fazeli, 2008). DON showed the highest ion suppression that could be the cause of the reduced sensitivity in terms of LOD of this mycotoxin. The inclusion of the methanol-freezing step in the sample preparation (by temporally physical retention during freezing) improved the sensitivity and the recovery in all matrices. Particularly, the difference was significant for polished rice ($p<0.01$) and WWN ($p<0.05$). In oat flakes no major improvement was observed, with the exception of ZEN, T-2 and HT-2 toxins. This step helped to reduce polar matrix interferences. However, the extraction of water soluble mycotoxins (i.e. $FB_1$) could be hampered. By constructing MMCC using representative real samples, matrix effects were substantially corrected. The recovery values determined using MMCC of spiked cereal matrices are presented in Table 4.4. Most recoveries were in agreement with the minimum requirements set in the regulation 401/2006/EC (70-110%) (European-Commission, 2006a). The achieved intra- and inter-day precisions for all cereal matrices are presented in Table 4.5 and the relative standard deviation values (% RSD) were according to the maximum percentages for quantitative methods set in the regulation 2002/657/EC (<20%) (European-Commission, 2002).

The type of matrix clearly had an impact on the variability of the results, in both precision and recovery. The highest variability was observed for wheat noodles, which could be attributed to their more complex composition, their higher protein and fat content or their higher degree of processing compared to the other matrices, or a combination of these three factors.

The validated method was further applied for the polished rice, oat flakes and wheat noodles (white and yellow). In addition, the method was also applied on paddy rice and the limits of detection and quantification are also presented in Table 4.2.

**Table 4.3.** Variation in signal suppression/enhancement (% SSE) amongst blank extracts of samples of polished rice, oat flakes, white wheat noodles (WWN) and yellow wheat noodles (YWN) spiked at 4 concentration levels in a range of 25-100 µg $kg^{-1}$.

| | % SSE calculated as "slope spiked extract/slope liquid standard slope × 100" | | | |
|---|---|---|---|---|
| **Mycotoxin** | **Polished rice** | **Oat flakes** | **WWN** | **YWN** |
| DON | 37 | 55 | 28 | 78 |
| $AFG_2$ | 67 | 77 | 87 | 82 |
| $AFG_1$ | 68 | 96 | 102 | 103 |
| $AFB_2$ | 61 | 60 | 63 | 60 |
| $AFB_1$ | 70 | 60 | 69 | 65 |
| HT-2 | 63 | 85 | 87 | 112 |
| $FB_1$ | 96 | 87 | 98 | 103 |
| T-2 | 73 | 69 | 84 | 98 |
| OTA | 91 | 66 | 79 | 82 |
| ZEN | 76 | 51 | 67 | 68 |

**Table 4.4.** Recovery (%) ± SD determined using matrix matched calibration curves of polished rice, oat flakes, white wheat noodles and yellow wheat noodles spiked at 3 concentration levels.

| % Recovery calculated as "((area-MMCC intercept)/MMCC slope) × (100/spiked concentration)" | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Spiking level [a]** | **DON** | **$AFG_2$** | **$AFG_1$** | **$AFB_2$** | **$AFB_1$** | **$HT_2$** | **$FB_1$** | **T-2** | **OTA** | **ZEN** |
| **Polished rice** | | | | | | | | | | |
| 0.5 | 89 ± 8 | 100 ± 3 | 99 ± 1 | 97 ± 4 | 100 ± 5 | 97 ± 4 | 112 ± 10 | 100 ± 3 | 94 ± 32 | 103 ± 7 |
| 1 | 111 ± 4 | 100 ± 4 | 101 ± 7 | 103 ± 3 | 100 ± 3 | 103 ± 6 | 88 ± 10 | 100 ± 6 | 96 ± 9 | 97 ± 14 |
| 1.5 | 96 ± 3 | 100 ± 5 | 100 ± 4 | 99 ± 3 | 100 ± 2 | 99 ± 3 | 96 ± 6 | 100 ± 2 | 101 ± 13 | 101 ± 10 |
| **Oat flakes** | | | | | | | | | | |
| 0.5 | 98 ± 4 | 97 ± 5 | 97 ± 5 | 100 ± 6 | 99 ± 4 | 111 ± 4 | 98 ± 9 | 96 ± 4 | 95 ± 9 | 93 ± 8 |
| 1 | 102 ± 6 | 103 ± 2 | 103 ± 2 | 100 ± 3 | 101 ± 3 | 97 ± 8 | 106 ± 3 | 104 ± 2 | 105 ± 8 | 107 ± 7 |
| 1.5 | 99 ± 4 | 99 ± 3 | 99 ± 1 | 100 ± 4 | 100 ± 2 | 105 ± 9 | 98 ± 5 | 99 ± 2 | 98 ± 8 | 98 ± 6 |
| **White wheat noodles** | | | | | | | | | | |
| 0.5 | 100 ± 17 | 113 ± 13 | 93 ± 27 | 93 ± 21 | 97 ± 25 | 107 ± 15 | 118 ± 22 | 109 ± 22 | 106 ± 13 | 102 ± 10 |
| 1 | 92 ± 15 | 93 ± 22 | 97 ± 21 | 88 ± 17 | 94 ± 15 | 93 ± 24 | 82 ± 14 | 85 ± 12 | 94 ± 21 | 105 ± 12 |
| 1.5 | 94 ± 10 | 95 ± 20 | 93 ± 22 | 104 ± 22 | 96 ± 15 | 102 ± 24 | 106 ± 14 | 96 ± 14 | 98 ± 15 | 98 ± 11 |
| **Yellow wheat noodles** | | | | | | | | | | |
| 0.5 | 94 ± 14 | 93 ± 10 | 101 ± 12 | 109 ± 11 | 96 ± 10 | 106 ± 12 | 116 ± 11 | 101 ± 12 | 102 ± 8 | 99 ± 20 |
| 1 | 106 ± 17 | 107 ± 22 | 99 ± 15 | 91 ± 6 | 100 ± 16 | 94 ± 21 | 81 ± 6 | 99 ± 17 | 98 ± 21 | 95 ± 9 |
| 1.5 | 98 ± 11 | 98 ± 12 | 100 ± 11 | 103 ± 11 | 99 ± 8 | 102 ± 18 | 101 ± 8 | 100 ± 13 | 101 ± 8 | 94 ± 13 |

[a] Fold-times the individual concentration of 640 µg $kg^{-1}$ for DON; 8 µg $kg^{-1}$ for $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$; 80 µg $kg^{-1}$ for HT-2 toxin and ZEN, 40 µg $kg^{-1}$ for T-2 toxin and OTA, and 200 µg $kg^{-1}$ for $FB_1$.

**Table 4.5.** Relative standard deviation (% RSD) of intra- and inter-day precision for polished rice, oat, white wheat noodles (WWN) and yellow wheat noodles (YWN) spiked at 3 concentration levels [a].

| Mycotoxin | Polished rice | | Oat flakes | | WWN | | YWN | |
|---|---|---|---|---|---|---|---|---|
| | Intra-day | Inter-day | Intra-day | Inter-day | Intra-day | Inter-day | Intra-day | Inter-day |
| DON | 4 | 7 | 5 | 10 | 16 | 20 | 15 | 19 |
| $AFG_2$ | 4 | 5 | 3 | 13 | 15 | 12 | 11 | 20 |
| $AFG_1$ | 4 | 16 | 2 | 16 | 18 | 15 | 16 | 19 |
| $AFB_2$ | 3 | 2 | 4 | 3 | 17 | 19 | 10 | 19 |
| $AFB_1$ | 3 | 6 | 3 | 13 | 15 | 17 | 12 | 18 |
| HT-2 | 4 | 8 | 12 | 16 | 15 | 14 | 15 | 20 |
| $FB_1$ | 11 | 17 | 4 | 8 | 14 | 11 | 15 | 19 |
| T-2 | 4 | 11 | 3 | 7 | 16 | 10 | 16 | 19 |
| OTA | 12 | 9 | 8 | 1 | 15 | 20 | 11 | 19 |
| ZEN | 9 | 8 | 7 | 4 | 10 | 4 | 14 | 16 |

[a] Spiked at 0.5-, 1- and 1.5-times the individual concentration of 640 µg $kg^{-1}$ for DON; 8 µg $kg^{-1}$ for $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$; 80 µg $kg^{-1}$ for HT-2 toxin and ZEN, 40 µg $kg^{-1}$ for T-2 toxin and OTA, and 200 µg $kg^{-1}$ for $FB_1$.

### 4.3.2. HPLC method performance

The calibration curves of OTA standard, both in pure solvent and in the cereal matrices, showed good linearity ($R^2 > 0.99$). The retention time of OTA was 10.7 ± 0.7 min for all cereals. The average recoveries in oat flakes, white and yellow noodles were 88 ± 4; 97 ± 8 and 103 ± 2%, respectively. Good average intra-day (%RSD=5.1) and inter-day (%RSD=5.2) precision were achieved. Both, recovery and precision values were also in agreement with EU criteria. The LOD's and LOQ's were 1.5 and 3 µg $kg^{-1}$ for oat flakes, and 1.9 and 3.8 µg $kg^{-1}$ for wheat noodles.

### 4.3.3. Mycotoxin co-occurrence in staple cereals in Ecuador

#### 4.3.3.1. Mycotoxin contamination in rice

Contamination rates, standard deviation of a sample proportion ($SD_p$), means, SD and ranges of all mycotoxins are presented in Table 4.6. Out of the 43 analyzed samples of paddy rice and 46 of polished rice, 21 (49%) and 3 (7%) samples, respectively, were contaminated at least with one mycotoxin. Polished rice was contaminated with $AFG_1$ or HT-2 toxin. Mycotoxin co-occurrence was found only in 3 samples of paddy rice (7%) (Guayas 67%, Los Rios 33%). The mycotoxin combinations found in this crop were: i) $AFG_1$, $AFB_1$ and $FB_1$ (33%) and ii) DON and $FB_1$ (67%) (data not shown). The levels of $AFB_1$ and $FB_1$ exceeded the established maximum limits for human consumption; however, paddy rice is not consumed as such. This contamination pattern

was in agreement with the possible mycotoxigenicity of the isolated fungal species of *Aspergillus* and *Fusarium* that had been previously described in Ecuadorian paddy rice (Pacin *et al.*, 2002). Moreover, low occurrences of DON and aflatoxins in Ecuadorian paddy rice had been formerly reported (Mühlemann *et al.*, 1997a, b).

In general, the rates of contamination in polished rice were considerably lower than in paddy rice, suggesting a substantial reduction of mycotoxin contamination with the milling process, as described in other studies (Sales and Yoshizawa, 2005; Ok *et al.*, 2009). On the other hand, the contamination with Fusarium toxins ($FB_1$, DON and HT-2) was higher for the rice from Los Rios than from Guayas province. Although, the findings suggested that the detected mycotoxins were mostly field-produced, the differences between Los Rios and Guayas might be also associated to the period between harvest and the milling process. The analyzed rice was harvested during the rainy season and the high humidity of paddy rice might lead to the growth of storage fungi, or proliferation of field fungi, and subsequently the rapid accumulation of mycotoxins (Sales and Yoshizawa, 2005). According to the survey applied during sampling, in both provinces similar frequencies of use of pesticides (58.3% Los Rios; 56.5% Guayas) and fertilizers (73.3% Los Rios; 74.2% Guayas) were reported. Furthermore, it was reported that the storage of paddy rice before milling was shorter in Guayas, with a minimum storage time of 1-5 days (70%) up to longer than 3 months (5%). In Los Rios the minimum storage time of 1-5 days was less frequently reported (53.1%) and, conversely, a storage time longer than 3 months was more frequent in this province (24%). Regarding management of mold infestation on the rice before milling, in Los Rios the major action reported was superficial mold cleaning before milling (38%), while disposal of the product (26%) and also superficial mold cleaning (23%) were the most common practices in Guayas province.

According to UNA (Ecuadorian National Storage Unit), the mill should accept fresh-harvested paddy rice (maximum 2 days old), with a maximum of 20% of moisture content and 5% of impurities (stones, insects, etc.). According to the survey, most supervisors referred that the incoming rice is usually accepted with higher moisture content (61% in Los Rios and 62% in Guayas) and impurities (65% in Los Rios and 46% in Guayas) than permitted. The reported average moisture content (Los Ríos 21% ± 6; Guayas 22% ± 5) and percentage of impurities (Los Ríos 8% ± 4; Guayas 8% ± 5) exceeded the permitted limits in both provinces without differing significantly ($P$=0.330 and $P$=0.760, respectively), This was in agreement with the higher mycotoxin occurrence observed in paddy rice. Hence, more severe quality controls of the incoming rice at the mill must be enforced to prevent post-harvest contamination in both, paddy and polished rice.

Additionally, the recommended maximum moisture content of polished rice is 14%. According to the survey, the moisture content of the final product is low enough to warrant safety storage for extended periods of time. The average moisture content of the

final product reported in Los Rios (11.5% ± 3) did not differ significantly from the reported in Guayas (11% ± 2) (P=0.215).

#### 4.3.3.2. Mycotoxin contamination in oat flakes

Contamination rates, standard deviation of a sample proportion ($SD_p$), means, SD and ranges of all mycotoxins are presented in Table 4.6. Out of the 42 analyzed samples (30 from Cuenca and 12 from Nabon), 9 samples (21%) were contaminated with one mycotoxin, being DON, OTA or $AFB_1$. The levels of $AFB_1$ and OTA exceeded the established maximum limits. No mycotoxin co-occurrence was observed in oat flakes. Since the LOD's of OTA of the multimycotoxin analysis by UHPLC/TOFMS were higher than the maximum permitted limits, additional analyses were performed by HPLC. Out of the 35 analyzed samples (31 from Cuenca and 4 from Nabon), 2 (6%) samples (1 from Cuenca and 1 from Nabon) were contaminated with OTA (mean=3.4 $\mu g\ kg^{-1}$ ± 0.3).

No data on mycotoxin occurrence in oat flakes are available in the Ecuadorian context. The low contamination rate reported could be related to the fact that most of the oat which is consumed in Ecuador is imported and the incoming oat should comply with the regulations enforced by the exporting countries; although in Ecuador no such controls are applied. Interestingly, it should be mentioned that 73% of the contaminated samples were those bought by weight from big sacks.

#### 4.3.3.3. Mycotoxin contamination in wheat noodles

Contamination rates, standard deviation of a sample proportion ($SD_p$), means, SD and ranges of all mycotoxins are presented in Table 4.6. Out of the 43 samples of white noodles (25 from Cuenca and 18 from Nabon), 15 samples (35%) were contaminated with DON and/or OTA (Figure 4.2). Co-occurrence of those mycotoxins was observed in 2 samples (5%) (data not shown). The levels of OTA exceeded largely the established maximum limits. Regarding yellow noodles, out of the 37 samples (27 from Cuenca and 10 from Nabon), 2 samples (5%) were contaminated with DON only. Additional analyses of OTA were performed by HPLC (29 samples of white and 30 samples of yellow noodles) and no contamination was detected in any of the samples.

The contamination of wheat-based products with DON and OTA is considered as a worldwide problem (Kushiro, 2008; Zaied *et al.*, 2009). However, the effect of processing into pasta largely contributes to the reduction of those mycotoxins (Kushiro, 2008; Duarte *et al.*, 2010; Gonzalez-Osnaya *et al.*, 2011). Furthermore, the yellow noodles manufacturing requires the inclusion of alkali salts and this might reduce the mycotoxin content, similarly to the reduction of aflatoxins and Fusarium toxins during nixtamalization of maize products. This could explain the lower mycotoxin occurrence observed in yellow noodles compared to the white ones. On the other hand, noodles in Ecuador are locally produced but most of the wheat (as durum wheat or flour) is

imported from countries were mycotoxin regulations are enforced. As well as for oat flakes, most of the contaminated samples (up to 80%) resulted to be those bought by weight from big sacks, situation that might contribute to fungi growth and subsequent mycotoxin production. Inadequate storage conditions could favor factors like moisture uptake and insect infestation; however, further research is necessary to identify the storage factors implicated in mycotoxin production at the temperate climate of the places where the samples were collected.

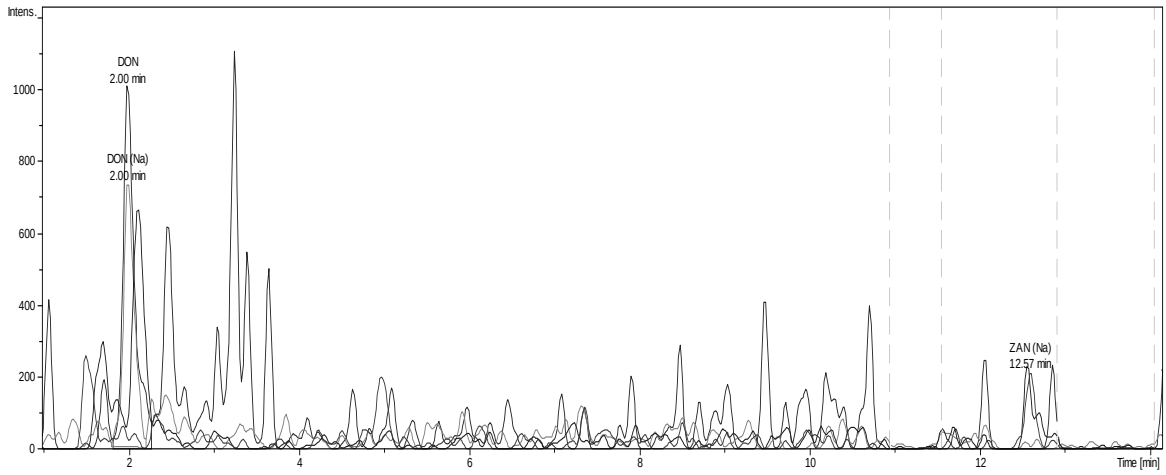



**Figure 4.2**. UHPLC/TOFMS chromatograms of natural contamination of DON in white noodles, spiked with ZAN for internal quality control.

**Table 4.6.** Contamination rates, standard deviation of a sample proportion ($SD_p$), means, SD and ranges of all mycotoxins in Ecuadorian staple cereals: polished and paddy rice, oat flakes, white and yellow wheat noodles, according to region of sample collection.

| Mycotoxin | Contamination rate (%,$SD_p$) | Region | Positive/total | Mean (µg $kg^{-1}$) [a] | SD | Range (µg $kg^{-1}$) |
|---|---|---|---|---|---|---|
| **Paddy rice (n=43)** | | | | | | |
| DON | 23%, $SD_p$ 6.4% | Guayas | 2/20 | 62.4 | 0.6 | 62 - 62.8 |
| | | Los Ríos | 8/23 | 79.9 | 21.5 | 43.9 - 102.4 |
| $AFG_2$ | 2%, $SD_p$ 2.3% | Guayas | 0/20 | - | - | - |
| | | Los Ríos | 1/23 | - | - | 3.3 [b] |
| $AFG_1$ | 2%, $SD_p$ 2.3% | Guayas | 1/20 | - | - | 63.7 [b] |
| | | Los Ríos | 0/23 | - | - | - |
| $AFB_1$ | 7%, $SD_p$ 3.9% | Guayas | 3/20 | 20.6 | 23.3 | 4.9 - 47.4 |
| | | Los Ríos | 0/23 | - | - | - |
| $FB_1$ | 23%, $SD_p$ 6.4% | Guayas | 3/20 | 40.4 | 16.3 | 22.6 - 54.3 |
| | | Los Ríos | 7/23 | 277 | 399.1 | 17.9 - 1146.4 |
| **Polished rice (n=46)** | | | | | | |
| $AFG_1$ | 2%, $SD_p$ 2.2% | Guayas | 0/20 | - | - | - |
| | | Los Ríos | 1/26 | - | - | 2 [b] |
| HT-2 | 4%, $SD_p$ 3.1 % | Guayas | 0/20 | - | - | - |
| | | Los Ríos | 2/26 | 32.8 | 9.6 | 26 - 39.5 |
| **Oat flakes (n=42)** | | | | | | |
| DON | 17%, $SD_p$ 5.8% | Cuenca | 4/30 | 50 | 17.2 | 32.2 - 69 |
| | | Nabon | 3/12 | 76.8 | 64.7 | 41.2 - 151.5 |
| $AFB_1$ | 2%, $SD_p$ 2.4% | Cuenca | 1/30 | - | - | 2.7 [b] |
| | | Nabon | 0/12 | - | - | - |
| OTA | 2%, $SD_p$ 2.4% | Cuenca | 0/30 | - | - | - |
| | | Nabon | 1/12 | - | - | 161.6 [b] |
| **White wheat noodles (n=43)** | | | | | | |
| DON | 33%, $SD_p$ 7.1% | Cuenca | 9/25 | 95.4 | 29.1 | 57.6 - 142.8 |
| | | Nabon | 5/18 | 120.5 | 58.2 | 87.4 - 224.2 |
| OTA | 5%, $SD_p$ 3.2% | Cuenca | 0/25 | - | - | - |
| | | Nabon | 2/18 | 60.8 | 45.7 | 28.5 - 93.1 |
| **Yellow wheat noodles (n=37)** | | | | | | |
| DON | 5%, $SD_p$ 3.7% | Cuenca | 2/27 | 85.6 | 1.9 | 84.2 - 86.9 |
| | | Nabon | 0/10 | - | - | - |

[a] Mean of positive samples
[b] Unique values

## 4.4. Conclusions

The co-occurrence of mycotoxins with the major health-concern in the most important Ecuadorian staple cereals (polished rice, oat flakes and wheat noodles) was reported. The analyses were performed by a reliable and sensitive UHPLC/TOFMS method that was developed and validated for those cereal matrices. Additional analyses of OTA in oat flakes and wheat noodles were performed by HPLC-FLD. Since no mycotoxin regulations are enforced in Ecuador, the obtained LOD's and LOQ's were compared with the European maximum permitted limits (regulation 2006/1881/EC) and only the LOD's of OTA in all cereals and AFB1 in yellow wheat noodles were higher than those limits. High rates of contamination were observed in white wheat noodles (33% DON, 5% OTA) and oat flakes (17% DON, 2% OTA and 2% $AFB_1$), and lower rates in polished rice (2% $AFG_1$ and 4% HT-2 toxin) and yellow noodles (5% DON). Although paddy rice is not consumed as such, it was also analyzed to evaluate pre-milling contamination (23% DON, 23% $FB_1$, 7% $AFB_1$, 2% $AFG_1$ and 2% $AFG_2$). Low rates of mycotoxin co-occurrence were observed only for white wheat noodles (5%) and paddy rice (7%). Although the levels of contamination of the studied mycotoxins were rather low, their occurrence in the assessed staple cereals may pose a hazard to public health considering their important role in the Ecuadorian diet. For follow-up studies, daily intake of those staple cereals must be estimated to support further risk exposure assessments. Furthermore, a retrospective analysis of emerging mycotoxins should be performed using the full spectral information generated by TOFMS.

# CHAPTER 5

## Multiple mycotoxin exposure and risk characterization of infants and young children by breastfeeding and complementary foods in the Ecuadorian highlands

# 5. Multiple mycotoxin exposure and risk characterization of infants and young children by breastfeeding and complementary foods in the Ecuadorian highlands

**Abstract**

In this chapter, the dietary exposure to co-occurring mycotoxins in children aged 0-23 months in the Ecuadorian highlands is presented. The exposure assessment was carried out in a sample of 923 children (320 rural and 603 urban) and based on the intake of cereal and cereal-based products (polished rice, wheat noodles and oat flakes) and breast milk. Contamination data on OTA, DON and HT-2 toxin in cereal and cereal products, and $AFM_1$ in breast milk were used for exposure assessment. A simple distribution approach followed by first order Monte Carlo simulation was adopted for assessment. This analysis was performed according to the children' feeding pattern: exclusively/predominantly breastfeeding; complementary feeding and weaning feeding stage. Risk characterization was carried out by comparing the estimated mean and percentiles of exposure with the tolerable daily intake (TDI) and estimating the margin of exposure (MOE). The exposure to $AFM_1$ through the consumption of breast milk in exclusively/predominantly breastfed children was considerably high in both areas. Similar MOE values (153 urban vs. 168 rural), proportion of the population above the PMTDI (provisional maximum tolerable daily intake) (49% urban vs. 44% rural) and a P99 which exceeded 3 times the PMTDI were observed in both areas. In children at complementary feeding stage, the most important health risk was the exposure to HT-2 through polished rice. The proportion of the population above the TDI was considerably higher in the rural area (15% vs. 5%) as well as the P99 (4 vs. 1.3 times above the TDI). At weaning stage, rural children were higher exposed to mycotoxins due to the higher consumption of cereal staple foods. The most important health risk was the exposure to HT-2 through polished rice (26%; P99 exceeding 5.5 times the TDI) and OTA through wheat noodles (15%; P99 exceeding 2 times the PTDI) and oat flakes (10%; P99 exceeding 2.4 times the PTDI). In the Ecuadorian context the likelihood of exposure to several mycotoxins at early childhood through multiple dietary sources should be monitored and the importance of further surveillance should be prioritized by risk managers.

## 5.1. Introduction

Mycotoxins are naturally occurring toxins produced by filamentous fungi under suitable temperature and humidity conditions on various foods and feeds before harvest, post-harvest or during processing and storage (Zain, 2011; Bezerra da Rocha *et al.*, 2014). Mycotoxins may affect diverse cellular processes and have a wide spectrum of toxicological effects such as hepatotoxic, nephrotoxic, neurotoxic, immunotoxic (Bhat *et al.*, 2010; Wild and Gong, 2010; Zain, 2011). In a single crop, several mycotoxins can be produced either from the same or from different fungal species. Combined toxic effects of mycotoxins could be antagonistic, additive or synergistic but actual knowledge about *in vivo* toxicological effects remains very limited (Speijers and Speijers, 2004; Shephard, 2008a; Capriotti *et al.*, 2012). Based on their adverse effects in human health, the most recognized mycotoxins are aflatoxins, fumonisins, ochratoxins, trichothecenes and zearalenone. (Bhat *et al.*, 2010; Wild and Gong, 2010; Zain, 2011).

Human exposure to mycotoxins occurs mostly through the intake of contaminated agricultural products or residues due to carry-over of metabolite products in animal products e.g. milk, meat or eggs (Zain, 2011; Capriotti *et al.*, 2012; Bezerra da Rocha *et al.*, 2014). Mycotoxins contaminate the diet of a large proportion of the world's population. In developing countries, the exposure to mycotoxins could be higher 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 (if available) in local markets instead of merely enforcement for trade purposes (Shephard, 2008b; Wild and Gong, 2010; Stoev, 2013). Moreover, children from developing countries are commonly exposed to rather monotonous cereal-based diets that are prone to mycotoxin contamination. Infants and young children are more susceptible than adults to the adverse effects of mycotoxins because of their higher intake/body weight ratio, higher metabolic rate and lower detoxification capacity. Consequently, the potential health risk for infants and young children is about three times higher than for adults (WHO, 2006a; Sherif *et al.*, 2009; Wild and Gong, 2010; Smith *et al.*, 2012).

Diet at early childhood is basically composed by breast milk and solid/semi-solid foods, namely complementary foods when are offered to the child together with breast milk, or weaning feeding stage when the child is no longer breastfed. The high and unique nutritional quality of breast milk is undoubted. Unfortunately, its safety can be endangered when mothers are exposed to mycotoxins through their diet. Particularly, maternal exposure to $AFB_1$ mainly leads to lactational transfer its hydroxylated metabolite $AFM_1$ (Galvano *et al.*, 2008; Andrade *et al.*, 2013). Furthermore, cereals are the most important staple foods in the world, and at the same time, mycotoxin contamination has been recognized as the main food safety problem in those products (Cheli *et al.*, 2014).

As described in *Chapter 3*, in Ecuador, cereals represent one of the major complementary and weaning staple foods for infants and young children. The co-occurrence of ten mycotoxins of health concern in polished rice, wheat noodles and oat flakes which are the main Ecuadorian cereal-based staple foods used for complementary and weaning feeding has been previously reported (*Chapter 4*). In general, the rates of mycotoxin contamination and co-occurrence in those foods were rather low. Only the levels of OTA in oat flakes and wheat noodles were above the maximum permitted limits in cereals set by the European Commission (European-Commission, 2006b). Despite the low levels of contamination, a risk of long-term low-level exposure could be expected when several mycotoxins affect basic staple foods (Kuiper-Goodman, 2004; Garcia-Cela *et al.*, 2012).

This chapter presents the part of the study that aimed at assessing the dietary exposure to co-occurring mycotoxins in children aged 0-23 months in the Ecuadorian highlands according to their feeding pattern. The exposures to HT-2 toxin through polished rice; DON and OTA through wheat noodles; DON and OTA through oat flakes, and $AFM_1$ through breast milk were assessed and compared to the maximum levels for oral chronic exposure established in order to prioritize risk management strategies.

## 5.2. Methodology

### 5.2.1. Aflatoxin $M_1$ in breast milk

#### 5.2.1.1. Chemicals and reagents

LC grade water, acetonitrile, methanol, phosphate-buffered saline (PBS) solution and standard solution of $AFM_1$ (10 µg $mL^{-1}$) in acetonitrile 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 solution was diluted with acetonitrile and aliquots were dried under a gentle stream of nitrogen and kept at -20°C. Standard stock solutions of 0.1 µg $mL^{-1}$ were prepared by reconstitution of the dried standard solution with a mixture of acetonitrile/water, 1:1 (v/v) and further dilutions were freshly prepared with the same solvent mixture.

#### 5.2.1.2. Sample collection

The analysis of $AFM_1$ was carried out in samples of breast milk from a total of 78 volunteer mothers in Nabon canton as part of a pilot study conducted from November 2012 to January 2013. Breast milk samples were obtained from lactating mothers by self-expression and collected into a sterile plastic container. Samples were transported at 4°C and then frozen within one day at -20°C until $AFM_1$ extraction and analysis.

#### 5.2.1.3. Sample preparation

Prior extraction, samples were skimmed with a mild-heat treatment in a water bath (Memmert, Munich, Germany) at 35-37°C for 10 min. followed by centrifugation for 15 min at 2403 g (Hettich EBA 20. Tuttlingen, Germany) and finally filtered (Whatman filter # 4) (Galvano *et al.*, 2008). A fixed volume (10 mL) of skimmed breast milk sample was applied to immunoaffinity clean-up columns (Easi-extract® Aflatoxin, R-Biopharm Rhône), which were previously brought to room temperature and conditioned with 3 mL of PBS solution. The sample of breast milk was passed at a flow rate of 2-3 mL $min^{-1}$. The column was washed with 10 mL of PBS solution followed by 10 mL of water at a flow rate of 5 mL $min^{-1}$. $AFM_1$ was eluted from the column with 1.5 mL of the mixture methanol/acetonitrile, 2:3 (v/v) passing through by gravity after 30 seconds of contact of the solvent with the cartridge. The eluate was back-flushed three times using a syringe and then air was pushed through the column. The collected eluate was finally diluted with 1 mL of water, which also passed through the column at a flow rate of 2-3 mL $min^{-1}$. The sample was filtered (0.45µm filter) and a volume of 200 µL was injected into the HPLC system.

#### 5.2.1.4. Instrumental parameters and analysis

$AFM_1$ was 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 (5µm, 4.6 x 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 of $AFM_1$ was performed by measurement of the peak areas at the $AFM_1$ retention time (4.1±0.04 min) and comparing them with a six-point calibration curve (1-15 ng $mL^{-1}$, $R^2 > 0.998$). Recovery experiments were performed in duplicate at 3 concentration levels (10, 15, 20 µg $L^{-1}$). The resultant $AFM_1$ concentration was finally corrected for the recovery (88±4%). The limit of detection was calculated based on a signal-to-noise ratio 3:1 (0.014 µg $L^{-1}$). 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 $AFM_1$ during 3 consecutive days. The procedure yielded an intra- and inter-day precision of 13.1% and 6.8%, respectively.

### 5.2.2. Mycotoxin co-occurrence in cereal and cereal-based products

Sampling plans, development of analytical methods and analysis have been previously described (*Chapter 4*). Briefly, samples of polished rice were collected from May to July 2010 (rainy season) from the biggest rice mills in Ecuador located at the lowlands of the coastal provinces of Guayas and Los Ríos. Samples of oat flakes, yellow-alkaline and white wheat noodles were collected from February-March 2010. Wheat noodles

were Fiocchetti type because this was the most commonly consumed type according to the dietary recalls. The samples of wheat noodles and oat flakes were bought at retail in open markets and supermarkets in Cuenca and Nabon. The 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 in 46 samples of paddy rice, 42 samples of oat flakes, 43 samples of white wheat noodles and 37 samples of yellow wheat noodles was determined using a UHPLC/TOFMS method. In addition, the occurrence of OTA in 35 samples of oat flakes, 29 samples of white wheat noodles and 30 samples of yellow wheat noodles was determined by HPLC with fluorescence detection.

No contamination above LOD of $AFB_2$, $AFG_2$, $FB_1$, ZEN, and T-2 toxin was found. Occurrence, standard deviation of the sample proportion ($SD_p$), number of samples >LOD, means in positive samples, SD and contamination ranges of all mycotoxins in all samples of polished rice, oat flakes, wheat noodles are presented in Table 5.1. $SD_p$ were calculated according to (Uyttendaele *et al.*, 2009).

**Table 5.1.** Occurrence, standard deviation of the sample proportion ($SD_p$), number of samples >LOD, means, SD and contamination ranges of all mycotoxins in samples of polished rice (from Ecuadorian rice mills), oat flakes and wheat noodles (from retails markets in Cuenca and Nabon, Ecuador) and breast milk (from mothers of Nabon, Ecuador).

| | Occurrence (%,$SD_p$) | Samples > LOD /total | Mean (µg $kg^{-1}$) [a] | SD | Range (µg $kg^{-1}$) |
|---|---|---|---|---|---|
| **Polished rice** | | | | | |
| HT-2 | 4%, $SD_p$ 3.1 % | 2/46 | 32.8 | 9.6 | 26 - 39.5 |
| $AFG_1$ | 2%, $SD_p$ 2.2% | 1/46 | - | - | 2 [d] |
| **Oat flakes** | | | | | |
| $AFB_1$ | 2%, $SD_p$ 2.4% | 1/42 | - | - | 2.7 [d] |
| DON | 17%, $SD_p$ 5.8% | 7/42 | 61.5 | 41.8 | 32.2 - 151.5 |
| OTA [b] | 4%, $SD_p$ 2.2% | 3/77 | 56.1 | 91.3 | 3.2-161.6 |
| **Wheat noodles** [c] | | | | | |
| DON | 20%, $SD_p$ 4.5% | 16/80 | 102.0 | 39.1 | 57.6 - 224.2 |
| OTA [b] | 1%, $SD_p$ 1.4% | 2/139 | 60.8 | 45.7 | 28.5 - 93.1 |
| **Breast milk** [e] | | | | | |
| $AFM_1$ | 12%, $SD_p$ 3.6% | 9/78 | 0.032 | 0.016 | 0.015-0.06 |

[a] Mean of positive samples

[b] Pooled contamination levels of OTA of first batch of samples analyzed by UHPLC/TOFMS and the second batch analyzed by HPLC-FLD.

[c] Pooled contamination levels found in white and yellow-alkaline wheat noodles. OTA was only found in white wheat noodles.

[d] Unique values.

[e] Expressed in µg $L^{-1}$

### 5.2.3. Food consumption data

Data on the consumption of rice, wheat noodles and oat flakes of a total of 998 children aged 0-23 months, 348 from a rural area (Nabon) and 650 from an urban area (Cuenca), were collected as part of a cross-sectional survey conducted from June to September 2008 to evaluate nutritional status in the Ecuadorian highlands. The methods of selection of the participants and study size had been previously described in *Chapter 2* and *Chapter 3*, respectively.

Individual consumption data were obtained from the primary child caregivers at their homes using 24-h dietary recall (24-hr) questionnaires. Duplicate 24-hr were carried out in the urban area, while a single 24-hr was carried in the rural area due to limited access

of these regions. The methods to estimate portion sizes had been previously detailed in *Chapter 3*. Briefly, 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. The estimation of breast milk intake was based on the average length (in minutes) of each suck that was recalled from the mothers and a proxy conversion factor of 6.15 grams of milk per minute of breastfeeding. Data entry was done in using the Lucille food intake software (Ghent-University, 2010) which allowed the quantitative estimation of food intake at ingredient level based on pre-set food composition databases.

The combined consumption of the cereal staples foods and breast milk on daily bases was assessed and prevalence of different combination patterns was reported. Children were grouped in three categories according their feeding pattern: **Group 1:** children being exclusively or predominantly breastfed (only water and water-based drinks, vitamins, minerals and medicines could be consumed besides breast milk); **Group 2:** children at complementary feeding stage (cereal and cereal products + breast milk); and **Group 3:** children at weaning stage (no breast milk intake).

Food consumption data were expressed as $kg.kg^{-1}$ body weight (bw) per day using individual weight data collected during surveying, as described in *Chapter 2*. The dietary exposure assessment was performed separately for children of the urban and rural area and accordingly their feeding pattern (three groups). In Cuenca, the individual usual dietary intake was determined from the duplicate 24-hr using the Multiple Source Method (MSM) program® (German-Institute-of-Human-Nutrition-Potsdam-Rehbrücke(DIfE), 2011), assuming an habitual consumption pattern for all intakes. The MSM program considers the intra-individual variability in consumption and is characterized by a two-part shrinkage technique applied to residuals of two regression models, one for the positive daily intake and one for the event of consumption. The MSM outputs for consumers were used to construct the distribution of consumption data. In Nabon, no usual dietary intake could be calculated using the MSM program as only one 24-hr was collected due to logistical constraints.

### 5.2.4. Dietary exposure assessment

Due to the nature of the obtained results on concentration data of mycotoxins i.e. low mycotoxin occurrence above LODs (*Chapter 3*), it was impossible to fit distributions on the contamination data and apply a probabilistic calculation approach. Therefore, a simple distribution approach was adopted to estimate the dietary exposure of mycotoxins. This consisted in the combination of a point estimate of the mycotoxin concentration (mean) with the distribution of individual consumption data (Lambe,

2002). The resulting distributions were modeled using first order Monte Carlo simulation based on 5,000 iterations.

For food contamination data, non-detected values (< LOD) were replaced by half of the limit of detection (medium bound value). The mean concentration was calculated for each mycotoxin. For the case of wheat noodles, contamination data of white and yellow-alkaline wheat noodles were pooled together because no distinction about the type of noodles was done during consumption data collection. Mycotoxins that occurred in only one of the samples (i.e. $AFG_1$ in polished rice and $AFB_1$ in oat flakes) were excluded from the exposure assessment.

Data on the consumption of cereal staples foods and breast milk on daily bases were fitted to normal probability distributions. The selection of the best-fit distributions was based on the lowest chi-square statistic and after inspection of probability–probability (P–P) plots. Distributions fitting and exposure assessment were carried out using the software package @Risk for Microsoft Excel version 6 (Palisade Corporation, US). Simulations were performed three times to ensure reliable convergence.

The results were reported as estimated exposure of each mycotoxin on daily bases considering the contribution of each of the assessed foods, all expressed as ng $kg^{-1}$ bw $day^{-1}$.

### 5.2.5. Risk characterization

The mean, P50, P75, P90, P97.5 and P99 of the dietary estimated exposure of the population were compared with the toxicological thresholds of OTA (PTDI=14 ng $kg^{-1}$ bw per day) (JECFA, 2007), HT-2 (TDI=100 ng $kg^{-1}$ bw per day) (EFSA, 2011a) and DON (TDI=1,000 ng $kg^{-1}$ bw per day) (SCF, 2002). In the case of $AFM_1$, the PMTDI (provisional maximum tolerable daily intake) for $AFB_1$ proposed by Kuiper-Goodman (Kuiper-Goodman, 1998) was considered (1 ng $kg^{-1}$ bw per day). The results were reported as percentages of the population at risk of exceeding the corresponding tolerable daily intake.

The risk of exposure was ranked by defining the margin of exposure (MOE) which is the ratio between the BMDL and the estimated mycotoxin exposure, where BMDL corresponds to the lower confidence limit of the bench mark dose, i.e. the dose which results in a predetermined level of adverse response (Pratt *et al.*, 2009). MOE values were calculated using the estimated mean exposure. BMDL values were selected from the experimental modeling in animals for most sensitive effect of the mycotoxin. The BMDL for DON corresponded to restriction of weight gain ($BMDL_{10}$=0.2 mg $kg^{-1}$ bw per day), for OTA corresponded to kidney adenoma and carcinoma ($BMDL_5$=0.05 mg $kg^{-1}$ bw per day) (Muri *et al.*, 2009), for $AFM_1$ corresponded to hepatocarcinoma ($BMDL_{10}$=170 ng $kg^{-1}$ bw per day; established for total aflatoxin content) (EFSA, 2007) and for HT-2 corresponded to the immunotoxicity and haematotoxicity in pigs

($BMDL_5$=0.01 mg $kg^{-1}$ bw per day) (EFSA, 2011a).

## 5.3. Results & Discussion

### 5.3.1. Exposure assessment & risk characterization

Occurrence, standard deviation of the sample proportion ($SD_p$), number of samples >LOD, means in positive samples, SD and contamination ranges of $AFM_1$ in breast milk is presented in Table 5.1. The input data for the exposure assessment i.e. mean mycotoxin concentration considering non-detected values as 0.5 LOD, and the best-fit distributions for consumption data are presented in Table 5.2.

Children from who data on body weight were not available were excluded from the analysis (n=75). In total, mycotoxin exposure was assessed for 603 children from the urban area (Cuenca) and 320 children from the rural area (Nabon). The daily estimated exposures of mycotoxins (mean, SD, P50, P75, P90, P95, P97.5 and P99) per food source in the consumer population of children of Cuenca and Nabon are presented in Table 5.3 and Table 5.4, respectively.

**Table 5.2.** Input data for exposure assessment per food source, feeding pattern category and region: a) Mycotoxin contamination data (considering non-detected values as 0.5 LOD): mean, minimum and maximum concentration, all expressed as µg $kg^{-1}$; b) Consumption data: number of consumers (n), mean, minimum and maximum and best-fit distribution function in Cuenca based on usual daily intake (MSM distribution) and in Nabon based on a single dietary 24-h recall, all expressed as kg $kg^{-1}$ bw $day^{-1}$.

**a. Mycotoxin contamination data (µg $kg^{-1}$)**

| | Mycotoxin | Mean | SD | Min | Max |
|---|---|---|---|---|---|
| Breast milk | $AFM_1$ | 0.01 | 0.01 | 0.007 | 0.06 |
| Polished rice | HT-2 | 11.2 | 4.9 | 10.2 | 39.6 |
| Wheat noodles | DON | 48.4 | 32.6 | 26.7 | 224.2 |
| | OTA | 4.3 | 8.3 | 1.0 | 93.1 |
| Oat flakes | DON | 21.3 | 24.3 | 13.2 | 151.6 |
| | OTA | 4.5 | 18.2 | 0.75 | 161.6 |

**b. Consumption data (kg.kg$^{-1}$ bw.day$^{-1}$)**

| | Region | n | Mean | Min | Max | Function |
|---|---|---|---|---|---|---|
| **Group 1: Exclusively/predominantly breastfed children** (Cuenca n=61; Nabon n=72) | | | | | | |
| Breast milk | Cuenca | 61 | 0.1129 | 0.0482 | 1.4861 | RiskLoglogistic(0,045723;0,055088;2,9398) |
| | Nabon | 72 | 0.1027 | -0.0317 | 0.4862 | RiskExtvalue(0,0751;0,047867) |
| **Group 2: Children at complementary feeding stage** (Cuenca n=303; Nabon n=163) | | | | | | |
| Breast milk | Cuenca | 277 | 0.0399 | 0.0039 | 0.4557 | RiskInvgauss(0,038588;0,05123;RiskShift(0,0013127)) |
| | Nabon | 157 | 0.0421 | 0.0028 | 3.5610 | RiskLoglogistic(0,0026908;0,023906;1,9084) |
| Polished rice | Cuenca | 187 | 0.0043 | -0.0005 | 0.0224 | RiskInvgauss(0,0059857;0,0365594;RiskShift(-0,0016837)) |
| | Nabon | 108 | 0.0067 | -0.0002 | 4.7864 | RiskLoglogistic(-0,00017662;0,0039244;2,0356) |
| Wheat noodles | Cuenca | 129 | 0.0015 | 0.0003 | 0.0180 | RiskLoglogistic(0,00027747;0,0010397;3,1036) |
| | Nabon | 67 | 0.0013 | 0.0001 | 0.0087 | RiskGamma(1,5609;0,00076642;RiskShift(0,00013515)) |
| Oat flakes | Cuenca | 99 | 0.0007 | -0.0001 | 0.0097 | RiskPearson5(5,237;0,004265;RiskShift(-0,00027156)) |
| | Nabon | 42 | 0.0009 | 0.0001 | 0.0083 | RiskExpon(0,0007518;RiskShift(0,00014346)) |
| **Group 3: Children at weaning stage** (Cuenca n=239; Nabon n=85) | | | | | | |
| Polished rice | Cuenca | 194 | 0.0048 | -0.0004 | 0.0203 | RiskExtvalue(0,0037857;0,0018007) |
| | Nabon | 73 | 0.0083 | 0.0002 | 0.3355 | RiskLoglogistic(0,00018807;0,0053361;2,0575) |
| Wheat noodles | Cuenca | 113 | 0.0015 | -0.0011 | 0.0043 | RiskLogistic(0,00145434;0,00027235) |
| | Nabon | 46 | 0.0019 | -0.0011 | 0.0105 | RiskExtvalue(0,0012714;0,0010788) |
| Oat flakes | Cuenca | 121 | 0.0013 | 0.0001 | 0.0112 | RiskPearson5(8,5727;0,013786;RiskShift(-0,0004997)) |
| | Nabon | 34 | 0.0014 | 0.0001 | 0.0219 | RiskInvgauss(0,0013677;0,0011607;RiskShift(0,0000533386)) |

**Table 5.3.** Estimated mycotoxin exposure per food source in children aged 0-23 months (n=603) in Cuenca (urban area) categorized according their feeding pattern, percentage of the population exceeding the tolerable daily intake (TDI) and margin of exposure (MOE). Mean, percentiles and TDI are expressed in ng $kg^{-1}$ bw $day^{-1}$.

| | Mean | SD | P50 | P75 | P90 | P95 | P97.5 | P99 | TDI [d] | % above TDI | MOE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Group 1** [a] | | | | | | | | | | | |
| $AFM_1$ in breast milk | 1.1 | 0.5 | 1.0 | 1.2 | 1.6 | 1.9 | 2.3 | 3.0 | 1 | 49% | 153 |
| **Group 2** [b] | | | | | | | | | | | |
| $AFM_1$ in breast milk | 0.4 | 0.3 | 0.3 | 0.5 | 0.8 | 1.0 | 1.3 | 1.7 | 1 | 6% | 431 |
| HT-2 in rice | 48.1 | 27.0 | 43.0 | 62.0 | 83.8 | 99.2 | 114.3 | 133.8 | 100 | 5% | 208 |
| DON in wheat noodles | 73.4 | 44.4 | 63.7 | 85.1 | 115.5 | 143.2 | 177.2 | 234.2 | 1000 | 0% | 2723 |
| OTA in wheat noodles | 6.6 | 4.0 | 5.7 | 7.6 | 10.3 | 12.8 | 15.9 | 21.0 | 14 | 4% | 7596 |
| DON in oat flakes | 15.6 | 11.8 | 12.7 | 19.6 | 29.1 | 37.0 | 45.8 | 59.1 | 1000 | 0% | 12809 |
| OTA in oat flakes | 3.3 | 2.5 | 2.7 | 4.2 | 6.2 | 7.9 | 9.8 | 12.6 | 14 | 0.7% | 15022 |
| **Group 3** [c] | | | | | | | | | | | |
| HT-2 in rice | 53.9 | 25.8 | 49.7 | 67.3 | 87.5 | 102.0 | 116.2 | 134.6 | 100 | 6% | 186 |
| DON in wheat noodles | 70.4 | 23.9 | 70.4 | 84.8 | 99.3 | 109.1 | 118.6 | 130.7 | 1000 | 0% | 2843 |
| OTA in wheat noodles | 6.3 | 2.1 | 6.3 | 7.6 | 8.9 | 9.8 | 10.6 | 11.7 | 14 | 0.1% | 7929 |
| DON in oat flakes | 28.1 | 15.1 | 24.9 | 34.7 | 46.8 | 56.1 | 65.8 | 79.5 | 1000 | 0% | 7125 |
| OTA in oat flakes | 6.0 | 3.2 | 5.3 | 7.4 | 10.0 | 12.0 | 14.0 | 17.0 | 14 | 3% | 8356 |

[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)

[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)

[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)

[d] PMTDI proposed for $AFB_1$ was used for risk characterization of $AFM_1$, PTDI (tolerable daily intake) was used for risk characterization of OTA.

**Table 5.4.** Estimated mycotoxin exposure per food source in children aged 0-23 months (n=320) in Nabon (rural area) categorized according their feeding pattern, percentage of the population exceeding the tolerable daily intake (TDI) and margin of exposure (MOE). Mean, percentiles and TDI are expressed in ng $kg^{-1}$ bw $day^{-1}$.

| | Mean | SD | P50 | P75 | P90 | P95 | P97.5 | P99 | TDI [d] | % above TDI | MOE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Group 1** [a] | | | | | | | | | | | |
| $AFM_1$ in breast milk | 1.0 | 0.6 | 0.9 | 1.3 | 1.8 | 2.1 | 2.5 | 2.9 | 1 | 44% | 168 |
| **Group 2** [b] | | | | | | | | | | | |
| $AFM_1$ in breast milk | 0.4 | 0.9 | 0.3 | 0.4 | 0.8 | 1.1 | 1.6 | 2.6 | 1 | 6% | 408 |
| HT-2 in rice | 65.6 | 117.6 | 41.9 | 73.2 | 127.0 | 184.2 | 263.0 | 416.1 | 100 | 15% | 152 |
| DON in wheat noodles | 64.4 | 46.3 | 52.6 | 85.6 | 126.0 | 155.3 | 183.9 | 221.3 | 1000 | 0% | 3105 |
| OTA in wheat noodles | 5.8 | 4.1 | 4.7 | 7.7 | 11.3 | 13.9 | 16.5 | 19.8 | 14 | 5% | 8661 |
| DON in oat flakes | 19.0 | 16.0 | 14.1 | 25.2 | 39.8 | 50.9 | 62.0 | 76.5 | 1000 | 0% | 10511 |
| OTA in oat flakes | 4.1 | 3.4 | 3.0 | 5.4 | 8.5 | 10.9 | 13.2 | 16.3 | 14 | 2% | 12327 |
| **Group 3** [c] | | | | | | | | | | | |
| HT-2 in rice | 92.7 | 140.4 | 61.7 | 103.8 | 175.5 | 251.4 | 355.4 | 553.0 | 100 | 26% | 108 |
| DON in wheat noodles | 91.6 | 66.9 | 80.6 | 126.5 | 178.9 | 216.3 | 253.2 | 301.2 | 1000 | 0% | 2183 |
| OTA in wheat noodles | 8.2 | 6.0 | 7.2 | 11.3 | 16.0 | 19.4 | 22.7 | 27.0 | 14 | 15% | 6089 |
| DON in oat flakes | 30.2 | 31.5 | 19.7 | 36.8 | 65.1 | 90.1 | 117.9 | 158.3 | 1000 | 0% | 6624 |
| OTA in oat flakes | 6.4 | 6.7 | 4.2 | 7.9 | 13.9 | 19.2 | 25.1 | 33.7 | 14 | 10% | 7768 |

[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)
[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)
[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)
[d] PMTDI proposed for $AFB_1$ was used for risk characterization of $AFM_1$, PTDI (tolerable daily intake) was used for risk characterization of OTA.

Breast milk was the only nourishing source of children from group 1; therefore all children could be exposed to $AFM_1$. No methodology for risk characterization of $AFM_1$ has been established yet (Caldas and Jardim, 2012), thus it was decided *a priori* to evaluate the risk using the PMTDI proposed for $AFB_1$ and the BMDL for the total content of aflatoxins. The dietary exposure to $AFM_1$ was similar in both settings (P99=3 ng $kg^{-1}$ bw $day^{-1}$ in Cuenca vs. 2.9 ng $kg^{-1}$ bw $day^{-1}$ in Nabon) as well as the proportion of the population above the PMTDI (49% vs. 44%, respectively). At this first stage of life (< 6 months of age), the bio-detoxification mechanisms are not as developed as in adults and then an inevitable chronic exposure could occur (El-Tras *et al.*, 2011; Dorne and Fink-Gremmels, 2013). Breastfeeding is an unequalled way of providing ideal food for the healthy growth and development of infants (WHO, 2002). However, the safety of breast milk can be hampered by the potential transfer of several chemicals. $AFM_1$ is the main hydroxylated metabolite of $AFB_1$ and can be detected in milk 12–24 hours after the first ingestion of foods contaminated with $AFB_1$. Besides $AFM_1$, other mycotoxins such as OTA, fumonisin $B_1$, ZEN and their metabolites can also be transferred into in breast milk (Navas *et al.*, 2005; Muñoz *et al.*, 2010; Ediage *et al.*, 2013). $AFM_1$ is an active metabolite of $AFB_1$ but its adverse effects on humans are still insufficiently described. Aflatoxins, as a group, have been designated by the IARC as human carcinogens (IARC/WHO, 2002). Based on animal studies, the potency of aflatoxin $M_1$ is considered to be 10% the potency of $AFB_1$ (FAO/WHO, 2001; Abdulrazzaq *et al.*, 2003; Sadeghi *et al.*, 2009).

At complementary feeding stage (group 2), semi-solid/solid foods are introduced to the child's diet. The proportion of the population exposed to $AFM_1$ above PMTDI was considerably lower than in group 1 in both areas (6%). However, the P99 in Nabon was similar than in group 1 and higher than in Cuenca (2.6 vs. 1.7 ng $kg^{-1}$ bw $day^{-1}$). The consumption of cereal-based staple foods was similar in both areas. Polished rice was the most frequently consumed cereal (60% in Cuenca and 64% in Nabon), followed by wheat noodles (40% in Cuenca and 41% in Nabon) and oat flakes (31% in Cuenca and 27% in Nabon). From those staple cereals, rice was a particularly important source of exposure to HT-2. The proportion of the population exposed to HT-2 above TDI was higher in the rural area (15% vs. 5%). In the rural area, the P99 for HT-2 was 4 times above the TDI; whereas in the urban area the P99 was 1.3 times the TDI. HT-2 is the

major deacetylated metabolite of T-2 toxin. HT-2 and T-2 toxins have potent immunotoxic and cytotoxic effects on animals, however limited information is available on the direct toxicity and adverse affects of HT-2 alone (Visconti *et al.*, 2005; Lattanzio *et al.*, 2009). HT-2, as other trichothecenes, has been designated by the IARC as non-human carcinogen (IARC/WHO, 1993, 2002).

On daily bases, children were usually consuming different combination of cereal-based staple foods and breast milk. As a consequence, multi-source exposure to mycotoxins could be expected. The combined consumption of staple cereals and breast milk was higher in Nabon compared to Cuenca (78% vs. 55%). In Cuenca, from ten different combinations of the analyzed foods, the most prevalent combination of consumption were breast milk + polished rice (17%), breast milk + polished rice + wheat noodles (10%), breast milk + polished rice + oat flakes (7%) and breast milk + wheat noodles (6%). In Nabon, there were eight different food combinations. The most prevalent were breast milk + polished rice (22%), breast milk + polished rice + wheat noodles (22%), breast milk + polished rice + oat flakes (11%) and the combination of all sources i.e. breast milk + polished rice + wheat noodles + oat flakes (7%).

At weaning stage (group 3), children were not longer breastfed, therefore the consumption of cereal-based foods and consequently the exposure to mycotoxins was higher than for group 2. In general, polished rice was the most consumed cereal (81% in Cuenca and 86% in Nabon). Also the frequency of wheat noodles consumption was comparable between settings (49% in Cuenca vs. 53% in Nabon). Oats flakes appeared to be consumed more frequently in the urban setting (51% in Cuenca vs. 41% in Nabon).

OTA has been designated by the IARC as possible human carcinogen (IARC/WHO, 2002). The exposure to OTA through oat flakes (14%. P99= 33.7 ng $kg^{-1}$ bw $day^{-1}$) and wheat noodles (15%. P99= 27 ng $kg^{-1}$ bw $day^{-1}$) became important for rural children at weaning feeding stage. The exposure (P99) to HT-2 through polished rice in the rural area was considerable higher than in the urban area (553.0 vs. 134.6 ng $kg^{-1}$ bw $day^{-1}$, respectively). Moreover, in this group the proportion of the rural population exposed to HT-2 above the TDI was larger than in group 2 (26%), which was not the case in the urban area. At this feeding stage, the proportional amount of rice consumed in the urban area was lower than at complementary feeding stage. This suggests that urban children had a more varied diet at this feeding stage which might have a diluting effect in the overall exposure to mycotoxins or which might lead to the introduction of other dietary sources that were not considered in this study.

Similarly to the children at complementary feeding stage (group 2), on daily bases the combined consumption of cereal-based staples was higher in Nabon than in Cuenca (68% vs. 44%). In Cuenca, the most prevalent food combinations were polished rice + oat flakes (19%), polished rice + wheat noodles (13%), polished rice + wheat noodles +

oat flakes (7%). In Nabon, the most prevalent combinations were polished rice + wheat noodles (30%), polished rice + oat flakes (18%), polished rice + wheat noodles + oat flakes (16%).

In this study, a high exposure to $AFM_1$ through breast milk was observed. However, it is noteworthy that a proxy PMTDI for $AFB_1$ was used to evaluate the population at risk. The exposure to $AFM_1$ was higher in the group of exclusively/predominantly breastfed children. Other studies showed a higher exposure to $AFM_1$ at complementary feeding stage (Ediage *et al.*, 2013) and at weaning stage (Gong *et al.*, 2003) in comparison with exclusive breastfeeding. This inconsistency could be explained by the different exposure assessment approach. In those studies, mycotoxin exposure was assessed by biomarkers monitoring and therefore the total exposure to each mycotoxin through multiple dietary sources is measured. Then besides $AFM_1$ in breast milk (Polychronaki *et al.*, 2007; Gürbay *et al.*, 2010), other sources such as non-human milk can contribute to the total exposure of $AFM_1$ at complementary or weaning feeding stage.

The limited data on exposure to mycotoxins of infants and young children from developing countries is mainly focused on corn-based staple foods due to different feeding patterns (Solovey *et al.*, 1999; Egal *et al.*, 2005; Kimanya *et al.*, 2009; Marin *et al.*, 2013). In South America, particular attention has been given to the exposure to aflatoxins through peanuts intake and fumonisins through maize intake (Brazil) and DON through bread intake (Argentina) (Pacin *et al.*, 2010; Caldas and Jardim, 2012). Regarding breast milk, similar occurrence and contamination level of $AFM_1$ in breast milk to those reported in this study were previously reported in samples from urban Brazilian mothers (Navas *et al.*, 2005). In addition, the exposure to OTA through breast milk of Chilean neonates was described, suggesting the importance of the evaluation of other mycotoxins besides $AFM_1$ in the region (Muñoz *et al.*, 2010).

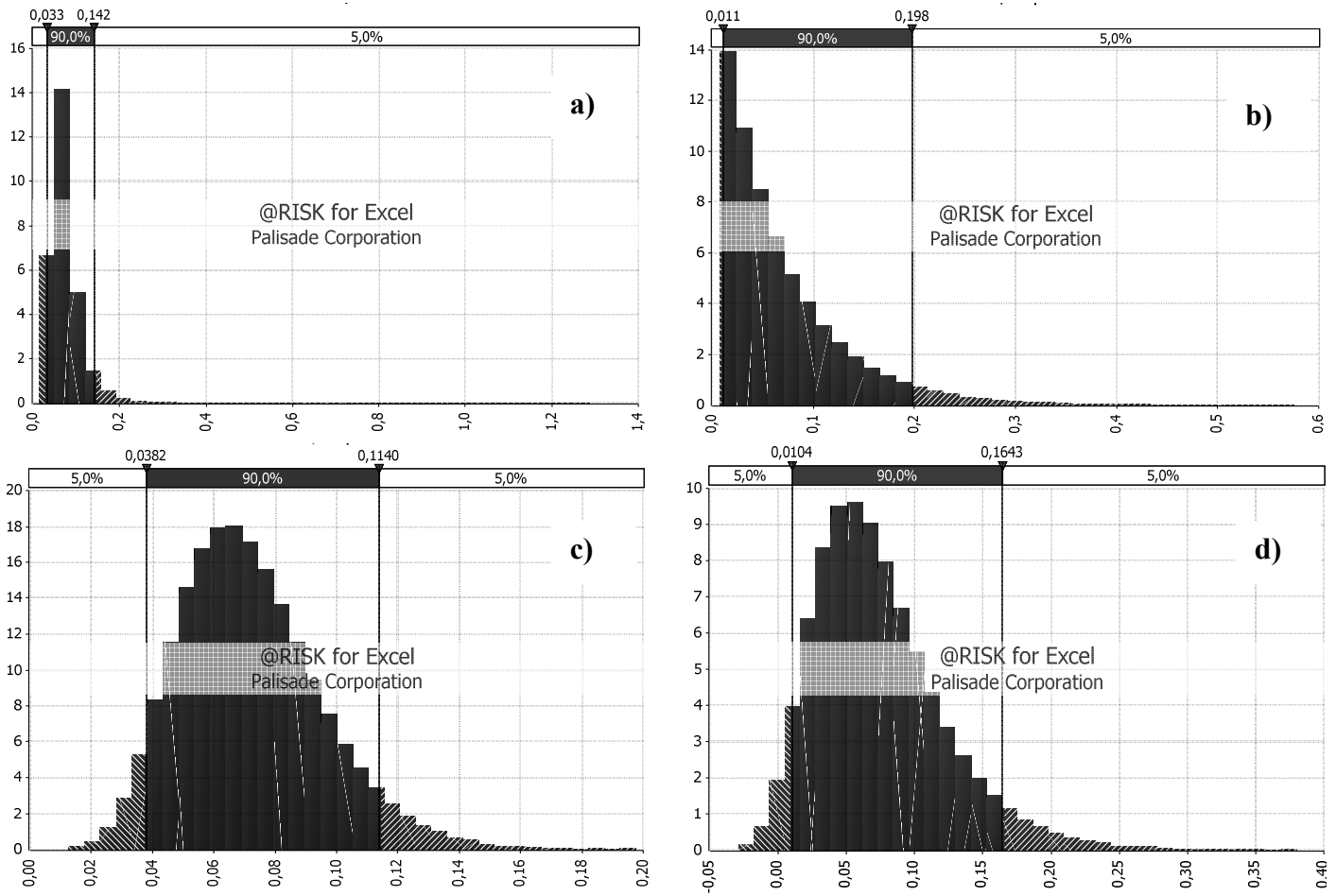
The Margin of Exposure (MOE) approach is conventionally applied for risk characterization of genotoxic and carcinogenic food contaminants. MOE value for oral exposure of 10,000 and above are of low concern from a public health point of view and therefore of low priority for risk management actions (EFSA, 2007; Pratt *et al.*, 2009). In this study, the MOEs were defined for all mycotoxins using the available BMDLs from animal studies. The obtained MOEs are presented in Tables 5.3 and Table 5.4. Most MOEs were below 10,000 except for the exposure to DON and OTA through oat flakes. Based on this ranking, the risk exposure to HT-2 through the consumption of polished rice and the exposure to $AFM_1$ through breast milk could be prioritized in this population. The application of the MOE approach to rank the risk of average exposures below the TDI might be less appropriate. That was the case of the exposure to DON through wheat noodles.

### 5.3.2. Usual intake of foods vs. single dietary assessment

The quality of the estimations from dietary exposure assessments depends highly on the

quality and uncertainties of the data used in each step of the process (Caldas and Jardim, 2012). A reference method for the assessment of food consumption is the use of duplicate 24-hr (Marin *et al.*, 2013). In Nabon, only a single 24-h dietary recall was carried out due to constraints in accessibility in the rural communities. In order to evaluate the degree of uncertainty due to this limitation in Nabon, the employment of duplicate vs. single 24-hr was compared using the Cuenca's dataset. For this purpose, distributions of consumption data of Cuenca considering only the first 24-hr were also constructed and the exposure to mycotoxins was estimated. As a result, no major variations in mean exposures were observed for both schemes. However, when comparing the tails of the distribution i.e. P90 - P99, the use of the distribution from a single dietary 24-hr yielded to an overestimation of the mycotoxin exposure on an average of 31% across the three feeding groups. The difference between the distributions is illustrated in Figure 5.1. As can be seen, the variance of the MSM distributions (adjusted for the intra-individual variability in food consumption) was lower than the variance of the distributions based on a single 24-h recall. Although this variation cannot be extrapolated, the overestimation resulting from this source of uncertainty must be considered when evaluating the exposure to mycotoxins estimated from a single dietary recall.

**Figure 5.1:** Distributions of the exposure of DON (in µg $kg^{-1}$ bw $day^{-1}$) by the consumption of wheat noodles in Cuenca: a) based on the MSM individual usual intake in group 2; b) based on the first 24-h recall in group 2, c) based on the MSM individual usual intake in group 3; d) based on the first 24-h recall in group 3.



Some additional limitations need to be addressed. First, the presented results must be interpreted carefully because the analyses were based on very low occurrence of mycotoxin contamination. In addition, since the simple distribution approach was adopted in this study, a worst-case scenario of exposure could be assumed. Secondly, the use of left censored, data i.e. replacement of non-detects concentration by the half of the LOD, might lead to produce a positive bias (Lambe, 2002; Kuiper-Goodman, 2004). Third, the contamination data on $AFM_1$ in breast milk were based only in samples from the rural area. The degree of contamination of breast milk might differ between urban and rural areas as suggested elsewhere (Mahdavi *et al.*, 2010). In addition, the degree of exposure to $AFM_1$ was evaluated using the toxicological cut-offs set for $AFB_1$. Subsequently, the health risk attributed to this exposure may be overestimated considering the difference in toxicological potency between both mycotoxins. Finally, no seasonal variations in mycotoxin occurrence were addressed. However, for the specific case of polished rice, samples were collected during the rainy season and therefore this may be considered the worst-case scenario for this staple food.

## 5.4. Conclusion

This is the first report of a dietary exposure assessment to mycotoxins through staple cereals and breast milk in Ecuadorian infant and young children. Although the relatively low occurrence and low contamination levels of mycotoxins in the assessed foods, the frequent consumption of these foods could result in a substantial chronic exposure due to the limited body detoxification capacity of this population. The exposure to $AFM_1$ and HT-2 through the consumption of breast milk and rice, respectively, raised particular concern in the studied population. Risk management options should be developed in order to control mycotoxin contamination in polished rice that was the most consumed cereal staple. Further evaluations of the maternal diet should be carried out in order to identify potential food sources of mycotoxin exposure. Subsequently, controlling this exposure, the risk of lactational transfer to the child could be reduced. The toxicological elucidation of short- and long-term adverse effects of multiple mycotoxin exposure on humans is still very limited. In this context, it was demonstrated that most young children usually consume different combinations of candidate food sources of mycotoxin contamination. Therefore, in Ecuador the likelihood of exposure to several mycotoxins at early childhood through multiple dietary sources should be monitored and further surveillance should be prioritized by risk managers.

# CHAPTER 6

## General discussion, conclusions and perspectives

# 6. General discussion, conclusions and perspectives

All children have the right of living in propitious environments that allow them to achieve their optimal growth and development. Such basic right forms one of the most important challenges for the public health sector given that it concerns a multi-sectorial problem.

The present study embraces the problems of nutritional failure and exposure to unsafe foods during the first 2 years of life in a rural (Nabon) and urban (Cuenca) canton at the Ecuadorian highlands.

We demonstrated that this population is facing the so-called double burden of malnutrition, i.e. the co-existing of over- and undernutrition. Stunting was major form of malnutrition in both ecologies. Even though, the prevalence of stunted children was considerably higher in the rural area (37.4% vs. 17.7%). The determinants of stunting in both settings were positively associated with the maternal nutritional status i.e. maternal BMI in Cuenca and maternal stunting in Nabon. The prevalence of overweight was slightly higher in Cuenca (12.4% vs. 8.8%), where it was positively associated to child morbidity and facility-based delivery. Wasting was the least prevalent form of malnutrition in both areas (7.1%). In Cuenca, wasting was positively associated with suboptimal child care, while in Nabon no association with overweight and wasting were observed.

Our results demonstrated differences in feeding patterns between rural and urban infants and young children. Those differences were particularly attributable to the quantity of food intake. Breastfeeding practices were generally better in the rural area, while in the urban area the intake of complementary and solid/semi-solid foods was considerably higher. The basic diet of rural and urban children was typically composed of plant-origin foods (cereal and cereal products, tubers, fruits and vegetables) and dairy products (especially cow milk). This basic diet was also characterized by remarkably low consumption of animal-source foods and vitamin A sources.

Several socio-demographic characteristics, such as facility-based delivery, health seeking behavior, maternal age, maternal education, maternal working status, number of children under 5 years at the household, maternal marital status and additional income from migration, were independently associated with higher food intake and better feeding practices. From all, maternal education and facility-based delivery were the defining factors of the differences in feeding patterns between the urban and the rural setting.

The safety of the rather monotonous child's diet was evaluated in terms of mycotoxin contamination, for which no regulations are enforced in Ecuador. The occurrence of ten

important mycotoxins of health concern (aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, aflatoxin $G_2$, ochratoxin A, deoxynivalenol, fumonisin $B_1$, zearalenone, HT-2 and T-2 toxins) in Ecuadorian cereal-based staple foods used for complementary and weaning feeding (rice, oat flakes and wheat noodles) was analyzed. Additionally, the occurrence of aflatoxin $M_1$ in breast milk was assessed. In general, low occurrence and co-occurrence of mycotoxins at moderately low levels of concentration was found. We suggested that a substantial reduction on mycotoxin content in polished rice could be achieved through an adequate milling process.

Finally, the exposure to co-occurring mycotoxins through cereal and cereal-based staple foods and breast milk was evaluated. This exposure assessment was performed according to children' feeding pattern: exclusively/predominantly breastfeeding; complementary feeding and weaning feeding stage. Our results demonstrated that the degree of exposure to several mycotoxins through multiple dietary sources differed according to the feeding stage and feeding pattern. Besides the health risk of $AFM_1$ in breast milk especially for exclusively breastfed children in both areas, the exposure to HT-2 in polished rice was particularly important for rural children.

### 6.1. Urban vs. rural settings

Poverty is both a cause and an outcome of poor human development (Victora *et al.*, 2008). The frame of this study was the Ecuadorian highlands, which has been historically characterized by high social and ethnic disparities (Larrea and Kawachi, 2005). Originally, the studied urban and rural areas were chosen based on the marked difference in socioeconomic status (SES), even though these ecologies are geographically rather close. Several limitations in accessibility and facilities at all levels made Nabon canton, and its 15 communities, an underprivileged environment. Our results confirmed partially the vulnerability of this rural population to continue facing generational nutritional problems such as stunting. On the other hand, the urban canton of Cuenca, which is the capital of the province and considered as the most prosperous city of the country, was neither the ideal environment for child nutrition. The array of SES disparities mainly due to the urbanization phenomenon was the main characteristics of the urban area. Those disparities might also lead to nutrition transition at early ages and subsequently to double burden of malnutrition in this setting. Most urban households were middle class (59%), while the urban poor households were the minority (11%). Particularly, a comparable vulnerability to adverse health outcomes between the urban-poor and the rural populations has been suggested (Harpham, 2009). This heterogeneous urban environment offered more variability in socio-demographic determinants compared to the rural environment. This constitutes a higher likelihood to observe associations with child nutritional status. It remains to be elucidated if other determinants, such as education, are only associated with child nutritional status in case a minimal socio-economic status is obtained. Such hypothesis would explain why very few candidate determinants were found associated with child nutritional status in

Nabon. In Cuenca, our results showed that maternal nutritional status, hygienic child care practices, response to child morbidity and facility-based delivery were particularly relevant in child nutritional outcomes.

Nowadays, female social status has been recognized as a key mediator of child nutrition (Ruel *et al.*, 2013). From our results, maternal education was the most important defining factor of the urban area and constituted the difference in child feeding practices between both settings. Higher maternal education levels contribute to better female enrollment into society. Subsequently, this female inclusion would improve household food security especially in urban areas (Ruel, 2000). Even though, the redistribution of the female time is demanded and its influence on child feeding practices is still uncertain.

## 6.2. Malnutrition outcomes & mycotoxin exposure

In this study, we identified the major child nutritional failures, their associations with socio-demographic determinants and their comparison between a rural and urban area. However, our results were based on cross-sectional data and therefore we discuss this more in an ecological way.

The reduction of child malnutrition rates is one of the main monitoring indicators of the Millennium Development Goals. Our results demonstrate that, in 2008, no major progress was achieved with regard to the baseline of 2004 (Lutter and Chaparro, 2008). From our results, stunting was the major growth failure in the children of both areas. Later in life, irreversible development deficiencies could be expected in this population (Grantham-McGregor *et al.*, 2007; Walker *et al.*, 2007; Black *et al.*, 2008). In the frame of the complex etiology of stunting, the associations with mycotoxins exposure has been suggested. Epidemiological studies have showed the correlation of aflatoxins and fumonisins exposure with impaired growth (Gong *et al.*, 2004; Kimanya *et al.*, 2010; Khlangwiset *et al.*, 2011; Turner, 2013). Nevertheless, the mechanisms through which mycotoxin cause impaired growth in humans and animals are not yet elucidated. A number of mechanisms have been proposed. Particularly, convergent metabolic pathways of aflatoxins, fumonisins and deoxynivalenol have been related to the pathogenesis of enteropathy, and consequently with impaired growth (Smith *et al.*, 2012). Aflatoxin $B_1$ is largely more toxic than other mycotoxins. Its association with impaired growth, including stunting, has been widely studied in animals (Khlangwiset *et al.*, 2011). The human exposure to aflatoxins could start since fetal stage and continue at early life through the intake of breast milk, both from maternal source (Abdulrazzaq *et al.*, 2003; Piekkola *et al.*, 2012). Thereafter, the exposure to several mycotoxins through different staple foods during complementary and weaning feeding stage tends to rise (Gong *et al.*, 2003; Polychronaki *et al.*, 2007; Ediage *et al.*, 2013). However, this depends on the degree of contamination of staple foods. In this study, we emphasized the high vulnerability of exclusive/predominantly breastfed infants to the adverse health

effects of mycotoxins. For those infants, breast milk is the only food source and at this age their detoxification capacity is still meager (Sherif *et al.*, 2009; Turner, 2013). Moreover, in this study the exposure to aflatoxin $M_1$ was assessed but other mycotoxins can also be transferred into breast milk (Andrade *et al.*, 2013; Ediage *et al.*, 2013). The exposure to HT-2 through the consumption of polished rice was important at complementary feeding stage. At weaning feeding stage, this exposure was considerably higher in the rural area. In general, our results showed that rural children were more exposed to mycotoxins than urban children because they were longer breastfed and consumed more rice. Moreover, it has been suggested that the concomitant problem and malnutrition could restrict even more the limited biochemical mechanisms for detoxification (Shephard, 2008b). In the rural area, the likelihood of long-term exposure to low-levels of mycotoxins due to their monotonous diet remained uncertain. In Cuenca, children usually consumed larger portions of foods but showed a more varied pattern of food consumed at complementary and weaning feeding stage. Hence, a more varied food intake could entail the consumption of risk-free foods or the exposure to different mycotoxins from varied food sources.

To establish the causality of mycotoxin exposure on linear growth, longitudinal studies must be carried out from delivery onwards. Even though, such studies demand more budget and long periods for following-up. In this study, the occurrence of ten mycotoxins of health concern was evaluated in the three main cereal staple foods. Nevertheless, the possible occurrence of mycotoxins in other food sources and the occurrence of several mycotoxins (other than aflatoxin $M_1$) in breast milk could contribute to the total mycotoxin exposure. Moreover, the co-occurrence of masked mycotoxins was not evaluated in this study, which could lead to an underestimation of the actual health risk. In addition, the seasonal variations in mycotoxin contamination remain unexplored.

## 6.3. Intervention alternatives

In order to achieve the best benefits throughout life, interventions to improve nutrition and child development should be focused on the first 1,000 days of life, i.e. the crucial period of pregnancy and the first 2 years of life (Ruel *et al.*, 2013). The outcomes of this study might lead to community-based intervention proposals in which different underlying causes of child malnutrition can be covered (Bhutta et al., 2013a; Ruel et al., 2013). In particular, the integration of nutritional counseling with food safety aspects should be considered. We emphasized the importance of facility-based delivery in nutritional outcomes. In general, health services could provide the appropriate frame to develop such nutrition-sensitive interventions. Based on our results, the intervention approaches should differ between the rural and urban area. This relies on the significant difference in maternal education between settings which was particularly important in child feeding patterns. Moreover, a positive association between maternal education with the perceptions about food safety has been described elsewhere (Sudershan et al.,

2008).

Nutrition-sensitive interventions could be also agriculture-oriented. Those approaches are closely related to raise agricultural productivity, to enhance poor households' income and improve access to high-quality diets, particularly in the rural areas (Ruel *et al.*, 2013). In this study, land's ownership and their use were not evaluated and therefore not major input could be provided in this context. However, attention should be given to the high consumption processed cereal products (wheat noodles and oat flakes) in the rural areas. From our observations, a limited amount of fresh crops produced locally at the household were consumed. Instead important amounts of dried cereals and cereal products were included in the diet. Then, dietary diversity would be reduced and the intake of carbohydrate-based diets would increase. The effect of these types of diet later in life should be evaluated in following-up studies.

Awareness of mycotoxin hazard is necessary in Ecuador. Health education in food safety should be culturally oriented depending on the target population (Sudershan et al., 2008). Some approaches for risk management have been proposed, particularly for developed countries (Baert et al., 2011; Baert et al., 2012). Those methodologies are based on solid frameworks which allow active interactions between drivers of food safety and stakeholders at different levels in society. In the Ecuadorian context, no food safety actions regarding mycotoxins in foods exist. Therefore, a pivotal step should comprise risk communication actions at policy levels prior to the design of sustainable intervention strategies.

Strategies towards the prevention of mycotoxin exposure could involve agricultural (pre- and post-harvest). The efficacy of agricultural approaches in mycotoxin mitigation has been widely evaluated (Williams *et al.*, 2004; Turner *et al.*, 2005; Garcia and Heredia, 2006; Wagacha and Muthomi, 2008; Foroud and Eudes, 2009; Bhat *et al.*, 2010; Caldas and Jardim, 2012). Of notice, affordable low-technology approaches such as hand sorting and sun-drying applied at farm level have shown a substantially reduction on aflatoxin exposure (Turner *et al.*, 2005). From our results, polished rice was the most important cereal-based staple (alone or in combination) in the Ecuadorian child's diet. Rice also constitutes one of the major staple foods later in life in the Ecuadorian population (Bermudez and Tucker, 2003; INEC, 2011a; Sanchez-Llaguno *et al.*, 2013). The importance of rice as a target food for agricultural-oriented interventions to control mycotoxin contamination relies on the fact that this cereal is regionally produced. Based on the difference in mycotoxin occurrence between paddy and polished rice, we also suggested that milling could substantially reduce the mycotoxin charge of paddy rice as observed in other studies (Sales and Yoshizawa, 2005; Ok *et al.*, 2009). Thereafter, more severe control of pre- and post- milling storage conditions must be enforced. The outcomes of this study could serve as starting point for mycotoxin awareness at governmental level and further enrollment of other food safety actors such as the Food and Agriculture Organization (FAO). Agricultural strategies must be

targeted to the rice-producing and rice-processing sectors which are located at the coastal region of Ecuador. Considering the importance of this staple cereal at national level, there is a constant governmental surveillance of the national storage of rice (due to trading demands). This could offer an excellent scene and opportunity for execution of mitigation strategies at post-milling stage. In addition, pre-harvest conditions, which were not within the scope of this study, should be assessed to clearly identify other critical points for mycotoxin contamination at that food chain stage.

On the other hand, both oat flakes and wheat noodles (as wheat durum or flour) are mainly imported from countries where mycotoxin regulations are enforced. However, the occurrence of ochratoxin A in those cereal products suggested a possible risk of mycotoxin production at post-harvest stages. Therefore, control measurements on the storage conditions of those foods should also be undertaken. In this regard, the private production sector is involved and the access may be very limited. Then, the establishment of legislation could drive to monitoring actions.

## 6.4. Additional considerations

The general limitations of this study should be addressed. First, the epidemiological component of this study was based on a cross-sectional design, which limited us to establish the causality to our findings. Even though, the observed associations could make this baseline study a valid starting point for future planning of longitudinal studies. Secondly, the extreme difference of SES between the rural and urban area was an obstacle for a better understanding of the inter-setting variation. Third, the external validity of our results is pending because no information on the covered issues is available in neighboring countries with comparable ecologies like Peru or Colombia. Fourth, the cross-sectional study was carried out in 2008. Despite the associations between socio-demographic determinants with child malnutrition would remain, to date several conditions at governmental level such as access to better health services or conditional cash transfer programs (Schady and Araujo, 2006) would demand additional evaluation. Similarly, at household level it might be necessary to evaluate consistent changes in living conditions such as the increasing attendance to child day-care centers in the urban area. Fifth, the assessment of feeding patterns and mycotoxin exposure in the rural area was based on a single dietary recall and therefore the inter-day variation was disregarded. As a consequence, we suggested a possible overestimation of the exposure to mycotoxins. Sixth, the low mycotoxin occurrence in the analyzed cereals was unexpected. This fortunate drawback did not allow performing a probabilistic assessment which deals better with uncertainties of the exposure. In addition, the exposure assessment was based on considerably low occurrence of mycotoxins which could mislead the actual exposure. On the other hand, another source of underestimation of the total mycotoxin exposure is the possible presence of bio-active mycotoxin metabolites (masked mycotoxins) which was not evaluated in this study. Finally, the effect of seasonal variations in mycotoxin contamination remains uncertain. However,

in the specific case of polished rice, sampling was carried out during the rainy season and therefore this represented the most favorable conditions for fungi growth and mycotoxin production.

## 6.5. Conclusions and future perspectives

Some long- and short-term actions against infant and young child malnutrition have been recognized as priorities in Latin American countries (Lutter and Chaparro, 2008). Long-term actions include the improvement of underlying determinants of child malnutrition, such as poverty, low levels of maternal education and high rates of morbidity. Feeding determinants were considered as short-terms actions and comprised early initiation and longer maintenance of breastfeeding, improvement of complementary feeding practices and the use of nutritious and safe foods.

The scope of this PhD thesis was the evaluation of the child nutritional status and the associations of child malnutrition with socio-demographic characteristics; as well as the evaluation of child feeding patterns and the assessment of dietary exposure to mycotoxins at early childhood in the Ecuadorian highlands. From this explorative study, we emphasized the persistent high rates of stunting and the considerable rates of overweight. Although this study was carried out in one province at the Southern highlands, our findings support the need of those long- and short-term actions to combat nutritional failures in the Ecuadorian context. We recommend that regionally-oriented intervention programs should be appropriately designed, implemented and monitored considering the identified key socio-demographic determinants of child malnutrition.

The food safety problem of mycotoxin contamination was also part of the scope of this study due to the association between chronic exposures with the genesis of stunting. In general, our results suggest a relative minor exposure through cereal staple foods and a considerable exposure through breast milk. However, those findings are subjected to the aforementioned limitations and further surveillance is recommended especially to address seasonal variations. We also recommend the development of agricultural management actions and policies to control mycotoxin contamination in staple cereals, in particular polished rice. Policy makers and governmental authorities are the target stakeholders to be contacted in order to begin the development of those actions.

From this explorative study, several research questions emerged and require further elucidation. First, the actual role of health services on child feeding practices and the rural community effect in breastfeeding practices need evaluation to be further addressed in intervention programs. Secondly, a multiscreening method in UHPLC/TOFMS was employed in this study. However the features of TOFMS for retrospective data analysis of non-target compounds were not completely explored. We recommend the evaluation of the full spectral information generated in this study to identify other mycotoxins or their metabolites, which were susceptible to the used

analytical conditions. Third, decontamination strategies towards the prevention of mycotoxin exposure could also be developed. In particular, some cooking processes such as alkaline treatments might represent affordable options at household level (Shapira, 2004; Williams *et al.*, 2004; Wild and Gong, 2010). Nevertheless, those processes could only contribute to a partial reduction of the total mycotoxin content in foodstuff and their efficacy, applicability and sustainability should be evaluated. Finally, the reduction of the exposure to mycotoxins of lactating mothers will lead to decrease the child's exposure to mycotoxins through breast milk consumptions. The assessment of the diet of lactating mothers in rural and urban settings is required to identify other candidate food sources of mycotoxins. Moreover, bio-monitoring could represent a valuable and broader alternative for exposure assessment in both, mothers and children. The measurement of biomarkers provides accurate information on the individual intake of mycotoxins, including toxicokinetics and toxicodynamics in the body (Wild and Gong, 2010; Ediage *et al.*, 2013). Therefore, explorative studies based on bio-monitoring could efficiently provide information to identify and prioritize the mycotoxins that represent important health risks for the community.

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# CURRICULUM VITAE

# CURRICULUM VITAE

Silvia Johana Ortiz Ulloa was born on January 6th 1983 in Cuenca, Ecuador. In 2007, she obtained the Bachelor´s degree of Biochemical Pharmacist at Cuenca University, Ecuador. During her undergraduate studies, she was a teaching assistant of Quantitative Chemical Analysis (2004-2006) at the Faculty of Chemical Sciences. She was granted as research assistant of the project "Nutrition and Food Security in the austral region of Ecuador, Clinical, Bromatological and Microbiological study". In the frame of this project, she carried out her undergraduate thesis work that was entitled "Determination of ferropenic anemia in children aged 5-12 years old in Cuenca (Yanuncay parish)" and the results this work were presented in an international conference.

Since her graduation, she has worked at the Cuenca University as a researcher of the VLIR-IUC project "Food, Nutrition and Health". In 2008, she was granted with a VLIR-IUC (sandwich) scholarship to follow a pre-doctoral training and then to start her PhD training in 2009 at the Faculty of Bioscience Engineering, Ghent University, Belgium. During this period, she has been professor assistant and senior lecturer at Cuenca University. She had supported the practical exercises of Food Chemistry at the Master in Nutrition and Rural Development and Master in Food Technology at Ghent University (2009). She has supervised the thesis work of two students of the Master Program in Nutrition and Rural Development (2010-2011) at Ghent University; two students of the Master Program in Industrial and Environmental Toxicology (2012-2013) at Cuenca University, one student of Master Program in Agro-Food Sciences (2013) at Tolima University (Colombia) and ten undergraduate theses at the Faculty of Chemical Sciences, School of Biochemistry & Pharmacy, at Cuenca University (2011-2013).

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## ORAL PRESENTATIONS

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- Ortiz J., 2010. Estado nutricional y patrones alimentarios en niños de 0-24 meses del Azuay. II Congreso de Alimentación y Nutrición Infantil. Organizers: University of San Francisco of Quito, FUNBASIC, IBFAN. Quito, Ecuador. September 15$^{th}$ 18$^{th}$, 2010.

## POSTER PRESENTATIONS

- Ortiz J., Wijaya S., Donoso S., Van Camp J., De Meulenaer B., Huybregts L., 2013. Predictors of infant overweight in a rural and urban area of the Ecuadorian highlands. IUNS 20$^{th}$ International Congress of Nutrition. Organizers: International Union of Nutritional Science (IUNS), Sociedad Española de Nutrición. **In:** Ann Nutr Metab, 63, 1005. Granada, Spain. September 15$^{th}$-20$^{th}$, 2013.

- Ortiz J., Mestdagh F., Donoso S., Van Camp J., De Meulenaer B., 2011. Rapid screening for mycotoxins in rice, oat and wheat noodles by UHPLC/TOFMS. 4$^{th}$ International Symposium Mycotoxins: Challenges and Perspectives. Organizers: MYTOX (Research association "Mycotoxins and toxigenic moulds"), Food2Know, Ghent University, Hogeschool Gent. Ghent, Belgium. May 24$^{th}$, 2011.

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KVCV Food Division (Royal Flemish Chemical Society), Ghent University. Merelbeke, Belgium. June 9th, 2011.

- Ortiz J., Ochoa A., Andrade S., Escobar P., Abril V., Astudillo G., Van Camp J., Donoso S., 2011. Pilot study to develop a food composition database for a Southern region of Ecuador. 9th International Food Data Conference. Organizers: Food and Agriculture Organization (FAO), International Network of Food Data Systems (INFOODS), European Food Information Resource (EuroFIR AISBL), Food and Health Network (FHN). In: Food composition and Sustainable Diets, editors: Wright D. & Finglas P., pg. 146. Norwich-UK. September 14-17th, 2011.

- Ortiz J., Álvarez R., 2008. Influencia de la anemia ferropénica en el crecimiento: caso de niños escolares de la parroquia Yanuncay. Cuenca-Ecuador, 2006-2007. 4th International Colloquium and 5th National Colloquium of Food and Nutrition Research. Organizers: CIAN (Research Center of Food and Nutrition), University of Antioquia. **In:** Revista Perspectivas en Nutrición Humana. Escuela de Nutrición y Dietética de la Universidad de Antioquia. ISSN 0124-4108, pg. 65. Medellín, Colombia. August 13th-15th, 2008.

## FELLOWSHIPS

- FoodComp 2008: EUROFIR course on production and use of food composition data in nutrition. Institute: EUROFIR; VUP Food Research Institute; WU Division of Human Nutrition. Bratislava, Slovakia. October 6th-27th, 2008.

- 4th CREATE training course: Human Resource Management in Teams-Relations, power and globalization. Institute: CREATE (part of the Marie Curie mobility programme), Young European Associated Researchers, SINTEF. Trondheim, Norway May 26th-June 2nd, 2010.

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