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Environmental and Food Systems Exposures during Lactation: Mycotoxins and Pesticides in human milk from Haryana, India

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Environmental and Food Systems Exposures during Lactation: Mycotoxins and Pesticides in human milk from Haryana, India

By

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Abstract

Environmental and Food Systems Exposures during Lactation: Mycotoxins and Pesticides in human milk from Haryana, India

By Rukshan Mehta

Exclusive breastfeeding in the first six months of life is recommended globally as breastmilk contains nutrients and immune factors essential for child growth and development. Breastmilk can also carry contaminants such as mycotoxins and pesticides. We aimed to examine the prevalence of these exposures in breastmilk and food samples from Haryana, India.

We developed and validated a method to quantify 6 aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2) and 2 Ochratoxins (A & B) and tested it on four different types of animal milk samples (n = 38) collected from southern India, using ultra-high-performance liquid chromatography tandem mass spectrometry. Method performance parameters were in accordance with USFDA guidelines and LOQs ranged between 15.6 and 156 pg/mL. Using this method, we quantified AFB_2 in goat milk (mean: 38 pg/mL), AFM_1 in cow (331 pg/mL), goat (406 pg/mL), and pasteurized milk (164 pg/mL), and 90% of cow, goat and pasteurized milk samples were above EU limits of 50 pg/mL.

Next, we quantified 8 co-occurring mycotoxins in human breastmilk (infants 2-4 months) samples (n = 100) using UHPLC-MS/MS and ELISA to detect aflatoxin B_1, fumonisin B_1, and deoxynivalenol in several food commodities (n = 298). To the best of our knowledge, we present the first study to quantify mycotoxins in breastmilk from India. We found low concentrations of AFM_1 in 41% (> LOD) of samples (median: 13.7; range: 3.9-1200 pg/mL. AFM_1 was detected above FSSAI regulatory limits (500 pg/mL) in 27% of animal milk samples (n = 30). Maternal consumption of breads was associated ($\beta = 0.32$; 95% CI: 0.22, 10.99, $p < 0.05$) with breastmilk AFM_1 exposure. AFB_1 was detected in several food commodities including wheat (median: 1.9 µg/kg; 0-196), rice (0 µg/kg; 0-195.6), flour (3.13 µg/kg; 0-214.9). FB_1 was detected in maize, pearl millet and sorghum. DON was not detected in our study. Estimated daily intakes for 80% and 100% of women exceeded PMTDI cut-offs for AFM_1 consumption via animal milk and AFB_1 consumption in rice and flour (mean: 75.81; range: 35.2-318.2 ng/kg bw/day), respectively. Calculated daily intakes for AFM_1 in breastmilk exceeded PMTDI (1 ng/kg bw/day) recommendations for 40% of infants (mean 1.63; range: 0.26-18.20 ng/kg bw/day).

The third objective was to quantify 53 organochlorine, organophosphate and pyrethroid pesticides in breastmilk samples (n = 75) using GC-MS and GC-ECD. We used 30 in-depth interviews and 9 focus group discussions to assess maternal and community perceptions, knowledge, attitudes and practices associated with pesticide use. We detected *p,p'-DDT* and *p,p'-DDE* in 4% (range: < LOQ-28 µg/L) and 5% (range: < LOQ-107 µg/L) of samples, respectively. Findings suggest gendered engagement in farming, which precludes pesticide and fertilizer handling and use by lactating women. Chemicals are widely used despite varying perceptions of them as both medicines and poison, recognizing their need to ensure food security.

Although we did not detect pesticide concentrations of significant public health risk, we did quantify mycotoxins in human milk and food samples. Our findings suggest a need for further investigation of exposures across the food chain and associated health impacts.

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Dedication

I dedicate this dissertation to my parents and sister, Baktavar, Viraf and Tushna Mehta, and to my grandparents. To my parents, thank you for encouraging and motivating me to pursue my dreams and for your unwavering support and trust. To my grandparents, Rhoda Mehta & Mahazarine and Keki Mehta, I love and miss you. Thank you for inspiring me in more ways than one. I hope I've made you proud.

List of abbreviations

AF	Aflatoxin
OT	Ochratoxin
FB	Fumonisins
DON	Deoxynivalenol
OC	Organochlorine pesticides
OP	Organophosphate pesticides
PMTDI	Provisional maximum tolerable daily intake
MRL	Maximum residue limit
MTL	Maximum tolerable limit
MAL	Maximum allowable level
ADI	Acceptable daily intake
ELISA	Enzyme linked immunosorbent assays
FSSAI	Food Safety and Standards Authority of India
USFDA	United States Food and Drug Administration
JECFA	Joint Expert Committee on Food Additives
IARC	International Agency for Research on Cancer
UHPLC/MS	Ultra-High-Performance Liquid Chromatography and Mass Spectrometry
SRM	Selected Reaction Monitoring
LOD	Limit of detection
LOQ	Limit of quantification
LQC	Lower quality control
MQC	Middle quality control
HQC	Highest quality control
DP	De-clustering potential
CXP	Exit potential
EP	Entrance potential
CE	Collision energy

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Chapter 1: Introduction

Global estimates suggest that 195 million children are stunted, with greatest burden in South Asia and Sub-Saharan Africa (Black, Victora et al., 2013). According to the National Family Health Survey-4, 38.4% of children in India, below the age of 5 years are stunted. A study by Danaei et al., (2016) shows that 32.5% of stunting can be attributed to fetal growth restriction and pre-term birth, 28% to maternal and child nutrition and infection and 22% to environmental factors (Danaei et al., 2016). Many nutrition programs have neglected the role of environmental factors. While unimproved sanitation, water and use of biomass fuels have been explored in relation to childhood stunting, other environmental factors remain unexamined (Danaei et al., 2016). Children who lack access to improved sanitation facilities (OR: 1.37, 95% CI: 1.33, 1.41), lack access to improved water (OR: 1.09, 95% CI: 1.06, 1.13) have higher odds of stunting. Additionally, children from households where biomass fuels are used also have higher odds of low birth weight (<2500 gms) (OR: 1.40, 95% CI: 1.26, 1.54) (Fink et al., 2011; Bonjour et al., 2013; Bruce et al., 2013; Wolf et al., 2013).

While there is growing global attention to the role of water, hygiene and sanitation, and household biomass fuels for cooking on child growth, there remain many unanswered questions including the contribution of the 'exposome' which includes other environmental factors such as mycotoxins and pesticides, to early growth faltering (Owino et al., 2016; Mapesa et al., 2016).

Mycotoxins are secondary metabolites of fungi that are detected in staple crops, milk and animal feed. Mycotoxin contamination may occur in the field, during harvest and post-harvest, in storage (Wu et al., 2014; Warth et al., 2016). The FAO estimates that 25% of the global food supply is impacted by mycotoxin contamination. A large survey (n > 19,000) of feed samples from across the world, which measured aflatoxins, fumonisins, deoxynivalenol, zearalenone and

ochratoxin A in feed samples found 72% were contaminated with at least one mycotoxin with co-contamination being common (Schatzmayr & Sterit, 2013).

Children are particularly susceptible to the effects of mycotoxins as these substances target rapidly growing cells (Prendergast & Humphrey, 2014). Consumption of mycotoxin-contaminated foods has been linked to low birth weight, stunting, deficits in cognitive development, increased susceptibility to disease and vaccine resistance; as well as chronic health conditions in adulthood such as cancer, inflammation and endocrine changes (Warth et al., 2016; Smith et al., 2015). Limited research suggests, mycotoxin exposure may be a cause of environmental enteropathy, which could increase susceptibility to malnutrition (Smith et al., 2012). Mycotoxin exposure during lactation may also alter breastmilk composition by causing changes to the inflammatory profile (Warth et al., 2016). Studies in Africa and Bangladesh, where mycotoxin levels in foods are high, have shown increased inflammatory profiles of human breastmilk and high prevalence of subclinical mastitis (Gomo et al., 2003; Willumsen et al., 2003). Higher concentrations of immune factors in milk including IL-8 have likewise been linked with child growth faltering (Filteau et al., 1999; Rasmussen et al., 2008). However, to the best of our knowledge; no studies have been able to directly link maternal dietary intake/mycotoxin exposure, breastmilk composition and child health outcome data. Thus, critical gaps remain in our understanding of the mechanisms by which mycotoxin exposure may contribute to early child growth failure.

Pesticides are commonly used in India and have been associated with adverse impacts on child development and increased risk of cancer (Abhilash & Singh, 2009; Chen et al., 2014; Shi et al., 2011; Eskenazi et al., 2007; Eskenazi et al., 2008; Rauh et al., 2011). Over 90% of exposure to pesticides occurs via dietary intake (Tyagi et al., 2015). Pesticides have also been

detected in breastmilk, however the association between pesticide exposure and infant growth faltering remain unclear. Some human epidemiological studies have shown pesticide exposure during pregnancy to be associated with several adverse birth outcomes, including low birth weight, shorter gestational length leading to pre-term births and birth anomalies (Boccolini et al., 2012; Larsen et al., 2017). Animal studies suggest that pesticide exposure can contribute to elevated levels of oxidative stress, inflammatory marker status including TNF-α, interleukin-6β and nuclear factor (NF)-κB (Ghasemi-Niri et al., 2016). Chronic exposure to pesticides may lead to a 'leaky gut', paralysis of the gut lining and increased permeability of the epithelial barrier of the small intestine particularly during weaning, when the digestive tract is immature (Condette et al., 2014). This animal data is suggestive of a potential link between pesticide exposure during lactation, impaired gut integrity and growth failure but human data is lacking (Condette et al., 2014). We were uniquely positioned to leverage samples from an existing NIH R21 study to address questions about the extent of mycotoxin and pesticide exposures in breastmilk and food samples from Haryana, India.

1.1 Specific Aims

The specific aims of this dissertation are listed below and visually represented in **Figure 1** which describes the ***pathways of exposure*** examined in this dissertation:

Aim 1) *To develop and validate a method to detect and quantify mycotoxins (AFs: AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2 & Ochratoxins: OTA, OTB) in animal milk samples.*

Chapter 3: We developed and validated an ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method to detect and quantify mycotoxins (6 AFs & 2 OTs) in animal milk. We apply this method for detection of mycotoxins, described in this chapter to human breastmilk samples described in subsequent aims.

Aim 2a) *To screen for and quantify Aflatoxin B_1, Fumonisin B_1 and Deoxynivalenol in n=298 food items including wheat, rice, maize, pearl millet, dried red chilies, groundnuts, sorghum, wheat flour, barley, fresh and commercial buffalo milk collected from rural and peri-urban Haryana. To determine whether location of procurement and season impact concentrations of mycotoxins in food items.*

Aim 2b) *To quantify 6 Aflatoxins and 2 Ochratoxins in a cross-sectional sample of n = 100 human breastmilk samples from Haryana, India collected over a period of 12 months.*

Aim 2c) *To describe determinants of maternal breastmilk mycotoxin exposure and characterize risk of exposure for mothers and infants using a deterministic assessment approach.*

Hypothesis: Exposure to mycotoxins in the food system of study communities will lead to concentrations of aflatoxin B_1, fumonisin B_1 and deoxynivalenol, above maximum regulatory limits in commonly consumed food items. This in turn will lead to elevated concentrations of mycotoxins in human breastmilk samples among lactating women.

Chapter 4: We quantified 6 AFs and 2 OTs in human milk samples using the UHPLC-MS/MS method developed as part of aim 1. Additionally, we used enzyme linked immunosorbent assays (ELISA) to quantify levels of AFB_1, FB_1 and DON in food items collected from study communities at three distinct levels, namely village, mid-retail and wholesale markets. Samples were collected from peri-urban and rural communities to capture differences based on distance to the nearest urban center and across three seasons, namely summer, monsoon and fall. TOBIT or censored regression analysis was used to understand sociodemographic, environmental and dietary determinants of breastmilk mycotoxin concentrations.
We conducted risk assessments by comparing estimated daily intakes of mycotoxins for mothers and children to provisional maximum tolerable daily intake (PMTDI) values established by JECFA/FAO and other regulatory agencies. We also assessed margins of exposure to understand the level of risk posed by these mycotoxins in the food system and breastmilk to the health of mothers and children.

Aim 3a) *To quantify organochlorine, organophosphate and pyrethroid pesticides in a cross-sectional sample of n = 75 human breastmilk samples from Haryana, India collected over a period of 12 months.*

Aim 3b) *To use qualitative methods to understand community and lactating women's perceptions of, knowledge, attitudes and practices associated with pesticide exposures.*

Hypothesis: Exposure to pesticides in study communities will lead to concentrations of OCs, OPs and pyrethroids above maximum residue limits (MRLs) in human breastmilk samples of lactating women.

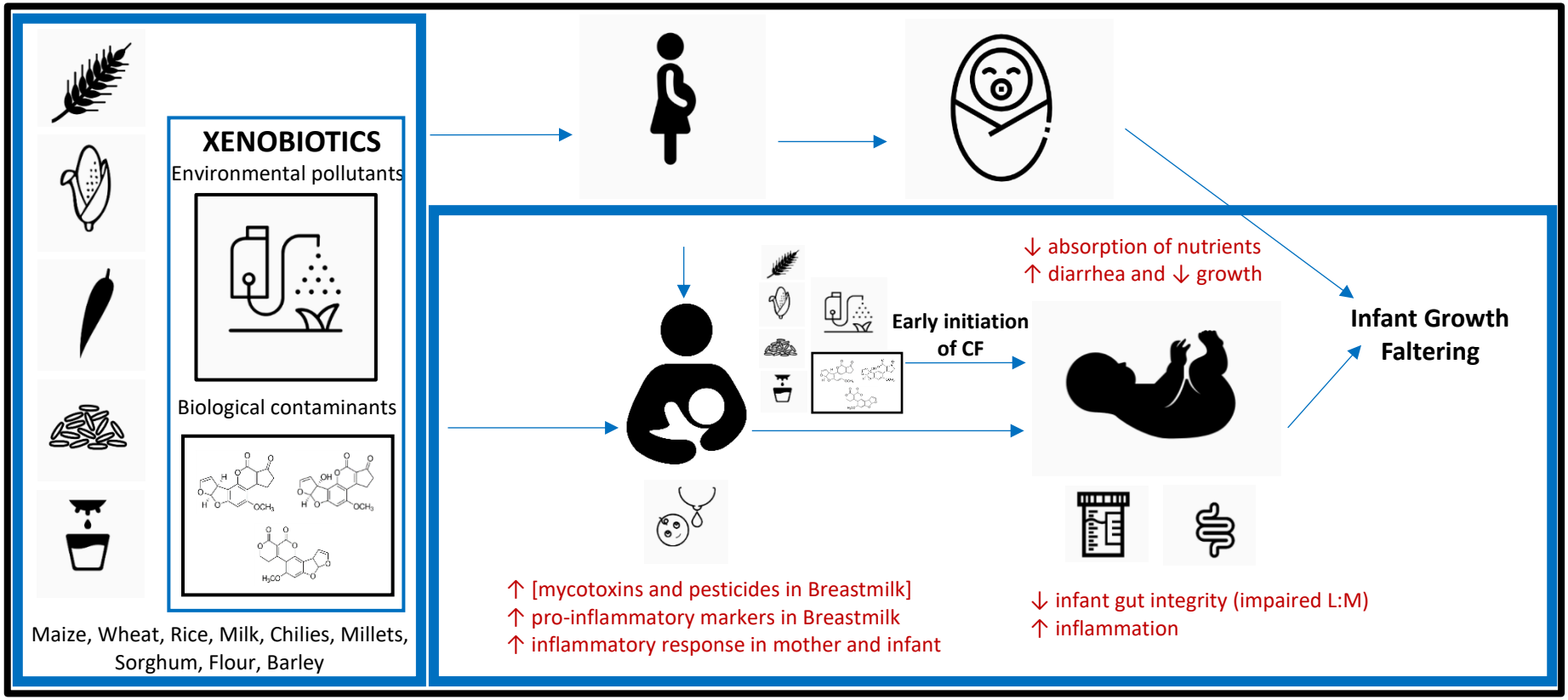
Chapter 5: We quantified 26 OC, 16 OP and 11 pyrethroid pesticides in human milk samples using gas chromatography mass spectrometry (GC-MS) for OCs and OPs and gas chromatography coupled to an electron capture detector (GC-ECD) for pyrethroids. We used qualitative methods including in-depth interviews (n = 30) with lactating women and focus group discussions (n = 9) with mothers-in law and older women, fathers and older men and government frontline workers (FLWs) in study communities to assess perceptions of and KAP with regards to pathways of pesticide exposures.

Aim 4) *To conduct exploratory path analyses to understand associations between maternal breastmilk concentrations of mycotoxins and pesticides, breastmilk inflammatory markers, infant gut integrity and early infant growth faltering.*

Hypothesis: Elevated breastmilk mycotoxins are associated with early infant growth faltering (LAZ < -1 SD) mediated by a pro-inflammatory breastmilk profile and impaired infant gut integrity.

Chapter 6: We used mediation path analysis to determine associations between breastmilk mycotoxin concentrations and several breastmilk inflammatory markers, namely i) pro-inflammatory cytokines: - interleukin-8 (IL-8), tumor necrosis factor – alpha (TNF-α); ii) antigen: - secretory IgA (sIgA); iii) anti-microbial: - Lactoferrin; and iv) elevated Na:K ratio, a measure of sub-clinical mastitis. These were regressed against a urinary measure of infant gut integrity, namely the ratio of sugars lactulose to mannitol (L:M). Finally, we examined associations between these pathways and measures of infant growth, namely length-for-age (LAZ), weight-for-age (WAZ) and weight-for-length z-scores (WFL).

Figure 1: Pathways and routes of mycotoxin and pesticide exposure for mothers and infants during pregnancy and lactation



[a]*Pathways*: consumption, occupational, handling of foods during preparation; *Routes*: inhalation, dermal, ingestion

Chapter 2: Literature review

This literature review begins with a discussion on child growth in the gestational period and first 6 months of life, focusing on the role of breastmilk and exclusive breastfeeding as the primary source of nutrition for infants postpartum. I will then discuss emerging literature on environmental enteric dysfunction (EED), what is known about the sub-clinical inflammatory condition and its association with early infant growth faltering and stunting. Next, I will delve into the literature on the role of two environmental and food systems exposures, namely *mycotoxins* and *pesticides* and what is known about their associations with adverse pregnancy outcomes from animal and human studies. Following this, I will discuss what is known about the presence of these contaminants in human breastmilk, and novel evidence from emerging studies on the association between these exposures and EED. I will also review methods for exposure assessment, biomarkers and analytical methods for detection of xenobiotics in human samples. To tie this literature review together, I will conclude by discussing evidence for the mechanisms linking mycotoxins and pesticides to EED and in turn infant growth faltering and stunting outcomes in early childhood.

2.1 Child growth

The period from conception to 2 years of life, widely referred to as the 'first 1000 days', is critical for human growth and development (Victora et al., 2008). Undernutrition among children in this period and beyond, is expressed in several forms including stunting (length-for-age z-score < -2SD of the World Health Organization Child Growth Standards median), wasting (weight-for-length z-score < -2SD of WHO Child Growth Standards median), underweight (weight-for-age z-score < -2SD of WHO Child Growth Standards median), and as deficiencies of vitamins and minerals (Victora et al., 2010). These forms of undernutrition frequently co-exist and children who demonstrate multiple forms of anthropometric failure are at increased risk of morbidity and mortality (Nandy et al., 2005; McDonald et al., 2013; Prendergast et al., 2014). Analysis of large datasets from Africa, Asia and Latin America have shown that children who have co-morbid stunting and wasting have three times higher risk of mortality (HR: 3.4, 95% CI: 2.6-4.3), with this risk increasing over 12-fold when compounded by underweight (HR: 12.3, 95% CI: 7.7-19.6) (McDonald et al., 2013; Prendergast et al., 2014).

Growth failure in the first two years of life is an important indicator of undernutrition at the cellular level and reflects widespread functional impairment and is associated with reduced adult height (Coly et al., 2006; Stein et al., 2010; Martorell & Zongrone, 2012). Growth failure begins *in utero* as fetal growth restriction which can lead to impaired postnatal child growth and results from poor quality diets and high burden of infection in pregnancy and during the first 2 years of life (Martorell & Zongrone, 2012; Solomons et al., 2015; De Onis & Branca, 2016. Additionally, 20% of stunting is estimated to have *in utero* origins (Prendergast & Humphrey, 2014).

Although the mechanisms underlying pre-natal growth restriction are not fully understood, it is well known that the nutrient requirements of mothers are higher during pregnancy (Dewey, 2016). Potential causes of fetal growth restriction (FGR) include low body mass index, reduced weight gain, micronutrient deficiencies in pregnancy and maternal infections (Bhutta et al., 2013; Christian et al., 2013). South Asia has the highest prevalence of FGR (term birth, small-for-gestational age), which is estimated to impact 27% of all births in low- and middle-income countries (Bhutta et al., 2013; Lee et al., 2013). In India, approximately half of all births have FGR (are small for gestational age, sex-specific birth-weight below 10^{th} percentile for gestational age of a reference standard) which contributes more than a third of the attributable fraction for stunting (Christian et al., 2013; Gonzalez-Casanova et al., 2017).

The nutrient needs of children under 2 years of age are also higher, in order to support growth and development. Insufficient gains in length/height and weight as a result of childhood undernutrition are associated with increased risk of morbidity and mortality, and can lead to impaired mental development, lowered physical and neuro-development, reduced capacity to learn at school and lowered earning potential in adulthood (Victora et al., 2008; Adair et al., 2013; Bhutta et al., 2013; Prendergast & Humphrey, 2014).

Maximal growth velocity in healthy infants is achieved between birth and 6 months of age, a period that is critical for long-term neurodevelopment (Pongchareon et al., 2012). Most growth faltering occurs between the ages of 3 and 18-24 months, often due to insufficiency in the quality and quantity of food provided to children (Victora et al., 2010).

Growth faltering leading to stunting is intergenerational. Stunting in itself is cyclical, where women who are stunted are more likely to have children who are stunted, resulting in a cycle of poverty and reduced human capital (Martorell & Zongrone, 2012; Prendergast &

Humphrey, 2014). Studies have shown associations between maternal and infant birthweight, where a 100-gram increase in maternal birthweight is shown to increase offspring birthweight by 10-20 g (Ramakrishnan et al., 1999). Additionally, mothers who are born small for gestational age (SGA) are at increased risk of birthing SGA infants (Farina et al., 2015; Klebanoff et al., 1997). Maternal height, birthweight, intrauterine growth restriction (IUGR), in addition to maternal weight-for-age and height-for-age z-scores at 2 years of age are also associated with birthweight of offspring infants (Victora et al., 2008). The mechanisms underlying the intergenerational effects of growth failure have biological etiologies and include genetics and epigenetics, metabolic programming and encephalopelvic disproportion (Martorell & Zongrone, 2012; LeRoy & Frongillo, 2019). The intergenerational transmission of poverty and cultural manifestations of the 'eating down syndrome' driven by a fear of birthing large babies are important factors also at play (Martorell & Zongrone, 2012).

Stunting is one form of undernutrition in children. Stunting represents a cumulative record of past and present growth restrictions and insults impacting a child's length, commonly caused by inadequacies in nutrition exacerbated by frequent infections such as diarrhea (Uauy, Kain & Corvalan, 2013). Estimates suggest that stunted children earn 20% less as adults, when compared to children who are not stunted (Grantham-McGregor et al., 2007). Additionally, according to the World Bank, a 1% loss in adult height due to stunting in childhood is associated with a loss of 1.4% in economic productivity (World Bank, 2006).

Globally, 21.9% of children under 5 years of age are stunted (WHO, 2019). According to the National Family Health Survey 2014-2015, 38.4% of children under the age of 5 years nationally and 34% in the north Indian state of Haryana are stunted. In Faridabad district, 29.7% of children under 5 years of age are stunted (NFHS-4). It is estimated that 20% of Indian infants

under 6 months of age are stunted (Patwari et al., 2013; Young et al., 2013; Menon et al., 2018).

The WHO conceptual framework on stunting has classified several systemic factors such as poor quality and access to healthcare and education, food insecurity characterized by lack of *adequate, fairly priced and safe foods*, improper access to clean water and sanitation, as important contributors to stunting. These factors in turn lead to proximal causes of stunting which include poor maternal nutrition during pregnancy and lactation, IUGR, inadequacies in the home environment and in complementary feeding, inadequate breastfeeding practices and recurrent clinical and subclinical infections (Stewart et al., 2013).

Studies from many global contexts and in India more specifically, have examined determinants of stunting. Some common predictors observed across countries include, household socioeconomic status and wealth index, lower levels of maternal education, increasing age of child, sex of child (male), prolonged duration of breastfeeding (> 12 months), low birth weight, mother's age (< 20 years), unimproved sources of drinking water, low maternal BMI (< 18.5), small for gestational age, diarrheal episodes, low paternal education, household food security, rural place of residence (Akombi et al., 2017; Biswas & Bose, 2010; Sinha, Bijalwan, Rohatgi & Kumar, 2018).

The 2013 Lancet series on maternal and child nutrition examined the effects of scaling up ten nutrition interventions namely, pre-conceptional folic acid supplementation or fortification, maternal balanced energy protein supplementation, maternal calcium supplementation, multiple micronutrient supplementation in pregnancy, promotion of breastfeeding, appropriate complementary feeding, vitamin A and preventive zinc supplementation in children 6-59 months of age, management of severe acute malnutrition (SAM), and management of moderate acute malnutrition (MAM). Bhutta et al., (2013) estimated that if these 10 nutrition interventions could

be scaled up from present levels to coverage of 90%, that stunting could be reduced by 20.3% (range: 10.2-28.9) (Bhutta et al., 2013).

More recently, fourteen programs which demonstrated reductions in stunting (children 0-59 months) were identified from 19 low- and middle-income countries. The average annual rate of reduction (AARR) varied between 0.6-8.4 and most commonly implemented interventions included nutrition education, counselling, growth monitoring and promotion, immunization, water, sanitation and hygiene, social safety net programs, all of which were considered highly effective at reducing stunting (AARR $\geq$ 3%) (Hossain et al., 2017).

Martorell and Young (2012), have noted that household factors including wealth in addition to maternal factors such as height, weight and age are associated with growth faltering. The nascent literature on growth faltering has examined other determinants and found factors such as elevated levels of anti-lipopolysaccharide (LPS) immunoglobulin A at 6 months of age, to predict greater declines in LAZ at 18 months of age (Syed et al., 2018). This is likely a result of systemic and enteric inflammation which is correlated with anti-LPS IgA levels. Sub-clinical mastitis (indicated by a milk Na:K ratio > 0.6), has also been associated with infant growth faltering (OR: 4.3, 95% CI: 1.1, 15.8) (Wren-Atilola et al., 2018). Solomons (2003), has noted that ~ 40% of variance in linear growth retardation is ascribed to diet, with the caveat that the phenomenon and related nutrition programming aimed at reducing this outcome, must examine interactions with the "total environment", particularly among those living in poverty.

Broadly, the causes of early (< 6 months of age) infant growth faltering in low resource settings remain unelucidated and more research is needed to understand the role and contribution of environmental exposures including mycotoxins and pesticides, and mechanisms leading to growth failure, to better design programs and interventions.

2.1.1 Exclusive breastfeeding and breastmilk composition

Exclusive Breastfeeding

Exclusive breastfeeding, defined as feeding infants only breastmilk is the 'gold standard' and one of the most important nutrition interventions in early infancy (Rollins et al., 2016). Exclusive breastfeeding is recommended up to the age of 6 months, but only between 30 to 37% of infants between the ages of 1-5 months are exclusively breastfed, globally and in low- and middle-income countries more specifically (Bhutta et al., 2013; Rollins et al., 2016). NFHS-4 data show that 50.3% and 36.5% of children under 6 months of age in the Indian state of Haryana and district of Faridabad, more specifically, are exclusively breastfed (NFHS-4, 2016).

Breastmilk contains nutrients essential for optimal development and is a dynamic fluid which evolves over the course of lactation to meet the needs of the growing infant (Lawrence, 1994; Bai et al., 2010). Exclusive breastfeeding has benefits including reduced morbidity and mortality (Black et al., 2008; Rollins et al., 2016) and improved cognition (WHO, 2013). Promotion of exclusive breastfeeding is a cost-effective policy tool, especially in resource-limited settings. Benefits of breastfeeding extend to maternal health and well-being and include reduced duration of postpartum bleeding, decreased risk of breast and ovarian cancers, and lowered risk of osteoporosis and increased birth-spacing (Godfrey et al., 2002; Das et al., 2016; Rollins et al., 2016).

Several determinants of optimal breastfeeding practices in India and other developing countries have been identified in the literature. These include, cultural barriers, maternal education, age, parity, poverty, social and caste status, work burden, poor hygiene and sanitation, availability and utilization of antenatal and postnatal care services, and knowledge and awareness of best practices including early initiation and exclusivity of breastfeeding (Behera et

al., 2015; Patel et al., 2010; Teka et al., 2015; Jeeson et al., 1989; Bhutta et al., 2010; Das et al., 2016). A study conducted in the Indian state of Bihar, found that exclusive breastfeeding is also associated with factors such as seasonality (Das et al., 2016). These authors noted that the odds of receiving only breastmilk during the previous day were significantly higher in winter months (aOR: 1.50, 95% CI = 1.37, 1.63), compared to summer. In addition, children who were nursed in winter months had higher odds of being exclusively breastfed for 6 months (aOR: 1.90, 95% CI = 1.43, 2.52), when compared to those nursed in the summer. Authors postulated that women's perceptions of children remaining thirsty in the warmer months, leading to worries about the child not being properly nourished prompt supplementation with liquid or semi-solid food (Das et al., 2016; Huffman et al., 1980). This effect is accentuated in the latter part of the first six-month period, as the child grows, with perceptions of nutritional needs getting more pronounced, alongside increased infant demands for feeding (Das et al., 2016). Sub-optimal breastfeeding practices are a recognized determinant of stunting (Dewey & Mayers, 2011; Black et al., 2013; Prendergast et al., 2013).

Breastmilk Composition

Human milk is ideal for infants under 6 months of age and is a rich source of micronutrients, macronutrients and other bioactive elements which perform essential functions. These functions include their roles as anti-infectious and anti-inflammatory agents, growth factors and prebiotics (Ballard & Morrow, 2012).

Human milk protein content is estimated to be between 8-10 g/L (Butte et al., 2001). Casein and whey are the major protein components of milk, with the ratio of whey to casein ranging between 80:20 in early lactation to 50:50 in late lactation (Lonnerdal et al., 2003). Lipids contribute 40-55% to the total energy of human milk and are impacted by the stage of lactation

(Wu et al., 2018). Most human milk lipids are present in the form of triglycerides (95%), however phospholipids (0.8%), cholesterol (0.5%) and other components also exist and the lipid components of milk are packaged into milk fat globules, where the phospholipids form the bulk of the membrane and triglycerides are located in the core (Andreas et al., 2015; Koletzko, 2016; Jensen, 1999).

Lactose is the primary carbohydrate of human milk (Wu et al., 2018). In addition, human milk is comprised of several different human milk oligosaccharides which differ in size, charge and abundance (Wu et al., 2018). The concentration of HMOs in mature milk is 5-20 g/L (Bode, 2012) and 23-130 different HMOs have been identified (German, 2008). HMOs are prebiotics and function to stimulate colonization of beneficial gut microflora (Barile, 2013). In addition, they function as signaling molecules, immune-modulators and play a role in brain function development (Jantscher-Krenn et al., 2012).

Human milk contains a rich source of vitamins and minerals needed for optimal growth and development of children. Important nutrients include minerals such as zinc, iodine, calcium, and iron; and vitamins such as B-vitamins, vitamin D, vitamin A (Wu et al., 2018; Daniels et al., 2019). These nutrients are essential to ensure child growth, immune system and brain development (Dror & Allen, 2018)

Essential inflammatory markers found in breastmilk include but are not limited to pro-inflammatory cytokines such as interleukin-8 (IL-8) and TNF-α (Ballard & Morrow, 2013). Antigens such as secretory IgA and antimicrobials such as lactoferrin are also present (Wagner et al., 1996). All these components serve to protect the infant against infection and inflammation, help development of the infant gut and ensure infant survival (Goldman, 2000). The individual roles of these inflammatory markers in breastmilk are explored in further detail in chapter 6.

Factors that can influence maternal breastmilk composition include parity and age, maternal BMI, health conditions including obesity, diabetes, cystic fibrosis, hypobetalipoproteinemia and hypercholesterolemia (Wu et al., 2018). Environmental factors including SES, season and region of residence, in addition to maternal diet can also impact breastmilk composition (Wu et al., 2018).

In addition to the many nutritive elements, several different environmental contaminants have also been detected and quantified in human milk. Persistent organic pollutants such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), in addition to organochlorine pesticides such as DDT, heavy metals such as lead, mercury, arsenic and cadmium, and other pesticides have been found in breastmilk samples from around the world (Abballe et al., 2008; Ulaszewska et al., 2011; Koyashiki et al., 2011; Sharma et al., 2014). All of these toxicants' bio-magnify in the food chain over time and breastfeeding infants are the final target of these contaminants (Mead, 2008). In addition to chemical xenobiotics, naturally occurring mycotoxins have also been found in human milk samples from across the globe (Coppa et al., 2019). These environmental exposures will be discussed extensively, in subsequent sections below.

2.1.2 Environmental Enteric Dysfunction

Environmental enteric dysfunction or environmental enteropathy is a generalized disturbance in the structure and function of the small intestine, resulting in blunting or atrophy of intestinal villi, infiltration of inflammatory cells, and crypt cell hyperplasia (Owino et al., 2016). Pathophysiological changes associated with EED include i) gut leakiness and permeability; ii) microbial translocation; iii) gut inflammation; iv) systemic inflammation; v) dysbiosis; and vi) nutrient malabsorption (Kosek et al., 2013; Kelly et al., 2016; Korpe et al., 2012; Krebs et al.,

2014; Denno et al., 2014). Damage to intestinal epithelial integrity and disruption of tight junctions result in defects in intestinal permeability, which can lead to translocation or non-physiological uptake into the bloodstream, of intestinal luminal contents, including microbes and microbial products (Marchetti et al., 2013. Translocation of microbes can mediate and cause systemic intestinal inflammation. Impaired intestinal permeability results in undernutrition and is also a cause of growth faltering among children living in poverty (Kosek et al., 2017). Growth faltering and stunting in turn are associated with subclinical intestinal inflammation (Campbell et al., 2004; Steiner et al., 1998). Persistent systemic inflammation can compromise growth by interrupting bone growth potential, impairing release of growth hormone binding proteins in the liver (Thayu et al., 2010), suppressing appetite and increasing metabolic requirements (Hibbert et al., 2005). An increase in gut permeability can also affect absorption and metabolism of amino acids, proteins, lipids, carbohydrates and other nutrients (Keusch et al., 2014). EED has also been associated with oral vaccine response failure (Naylor et al., 2015).

Risk factors for EED are broadly categorized into factors of: i) systemic and social origin including poverty, poor hygiene and sanitation, nutrient deficiencies and food insecurity; ii) biological mechanisms which result in pathophysiology of the small intestine including enteric infections, and recurrent enteric infections which result in dysbiosis in the microbiota which colonize the duodeno-jejunum resulting in small intestinal bacterial overgrowth (SIBO) and a proinflammatory microbial community (Vonaesch et al., 2018); iii) finally pathophysiological outcomes of changes to the small intestine which include recurrent and persistent diarrhea, intestinal inflammation with and without diarrhea and stunting (Denno et al., 2016; Vonaesch et al., 2018). Additional risk factors include close contact with animals and frequent geophagy (Keusch et al., 2014; Crane et al., 2015; George et al., 2015; Semba et al., 2016). EED causes 5%

less carbohydrate absorption, 15% less protein absorption, as well as malabsorption of zinc and vitamin B12 (Korpe & Petri, 2012). Damage caused by EED allows infectious agents and other harmful substances to be absorbed by the body which in turn leads to chronic inflammation and diversion of essential nutrients away from normal growth, and also causes anemia (Prendergast et al., 2014; Kosek et al., 2014). Seminal longitudinal studies conducted in the Gambia have shown that > 40% of linear growth failure is attributable to EED (Lunn et al., 1991).

Biomarkers of EED

Several studies have been conducted on EED in recent decades with the aim to further elucidate the condition. Gold standard methods for detection of EED involve gut biopsies or intestinal aspirates, which are invasive and not feasible to acquire in field settings (Prendergast et al., 2015). Several noninvasive biomarker-based tests have been used to assess gut permeability and intestinal absorption. The most commonly used measures include **lactulose: mannitol (L:M)** ratio which is measured in urine and involves a dual-sugar absorption test, where a higher L:M ratio indicates greater gut permeability (no EED = L:M ratio ≤ 0.15; moderate EED = $0.15 < L:M < 0.45$; severe EED = L:M ≥ 0.45) (Ordiz et al., 2016). Additional biomarkers of EED in biological matrices such as feces include **myeloperoxidase (MPO),** which is an indicator of neutrophil activity in the intestinal mucosa, **neopterin (NEO),** a biomarker of T-helper cell activity and **alpha-1-antitrypsin (AAT),** a marker of protein loss and intestinal permeability (Kosek & MAL-ED Network Investigators, 2017).

The Malnutrition and Enteric Disease (MAL-ED) study which used standardized methods to assess gut biomarkers in 8 birth cohorts across 3 continents confirmed that this condition is common among young children (birth – 2 years) living in poverty (Kosek et al., 2013). The study was conducted in Bangladesh, Nepal, Pakistan, Brazil, Peru, South Africa and Tanzania (MAL-

ED Network Investigators, 2017). Children in the MAL-ED trials were enrolled at birth and followed up to 24 months of age. Samples were collected from children in two age groups, (4-11 mo.) and (12-21 mo.). Results from the MAL-ED study have shown the presence of MPO at concentrations 5-10x higher in the 8 cohort countries when compared to the USA. Additionally, AAT has been found at twice the concentrations and elevated levels of AGP and intestinal permeability (L:M, 2-3x higher) have also been noted when compared to healthy populations (Kremer et al., 1988, Kosek et al., 2017). Results from this study also indicate that growth velocities are lower in these settings leading to an increased prevalence of stunting by the age of two years (Kosek et al., 2017).

Using the L:M ratio test, studies have shown children with higher gut permeability to have alterations in serum metabolites, which include lower serum phosphatidylcholines, lysophosphatidylcholines and sphingomyelins, lower serum tryptophan, ornithine and citrulline and higher serum glutamate, taurine and serotonin (Semba, 2016). Perturbations in these pathways may in turn be associated with adverse consequences for child health and growth. Additional metabolomics-based studies conducted on children with EED have also shown secondary carnitine deficiency to be widespread, particularly in developing country contexts, where intake of animal source foods is low and lysine is a limiting amino acid in the diet (Semba et al., 2017). Carnitine is a conditionally essential nutrient which plays an important role in fatty acid metabolism and energy production, and its deficiency is associated with EED (Semba et al., 2017). These authors have also suggested that children with EED may have metabolic alterations associated with an abnormal gut microbiome, leading to an increase in metabolism of dietary polyphenols, phenylalanine and tyrosine (Semba et al., 2017).

Studies of maternal enteropathy have found that higher levels of maternal serum concentrations of anti-flagellin and anti-LPS Igs (measured at 18 weeks of gestation) are associated with shorter gestation, adverse birth outcomes, and reduced infant length at birth (Lauer et al., 2018). Aflatoxin mediated enteropathy and exposure to chemical toxicants such as pesticides and drugs have also been implicated as potential causes of EED, however further research is needed to understand pathways and mechanisms whereby these xenobiotics may exert their impact on infant growth faltering and stunting (Smith et al., 2012; Mapesa et al., 2016).

2.2 Mycotoxins

Mycotoxins are secondary metabolites of fungi that are produced by mold that contaminate all stages of the food chain and are toxic and carcinogenic to humans (Raiola et al., 2015). Mycotoxigenic growth is exacerbated due to weather conditions, insect and pest damage, poor harvest and post-harvest practices including storage of crops. This may lead to widespread human illness. It is estimated that 25% of the global food supply is impacted by mycotoxin contamination caused by mold or fungi (Marin et al., 2013). Over 300 different mycotoxins have been identified, of which Aflatoxins, Ochratoxins, Fumonisins and Deoxynivalenol are carcinogenic and known risks to public health. Agro-climatic conditions including high temperatures and humidity levels, particularly in monsoon season, inadequate storage facilities for cereals and produce, reliance on subsistence farming and limited monitoring and enforcement of regulations in local markets increase susceptibility to growth of mycotoxigenic fungi and mold (Shepard et al., 2010; Wild & Gong, 2010).

Children are particularly vulnerable to the negative health effects of mycotoxins as they consume more food than adults per kg body weight (Health Council of the Netherlands, 2004), resulting in higher exposures. Young children under the age of 1 are vulnerable as their

enzymatic activity and ability to break down chemical compounds is lower when compared to adults (Raiola et al., 2015). Insufficient kidney function and hepatic metabolism, in addition to variations in absorption, distribution and excretion of environmental toxicants particularly in the first 6 months of life increase vulnerability of infants (Makri et al., 2004; Munoz et al., 2014). In addition, infants have a glomerular filtration rate that is 1/3 lower than adults, alcohol dehydrogenase in the liver is also not fully developed, compounded by lower levels of de-hydroxylation and oxidation enzymes which are important for hepatic detoxification (Postupolski et al., 2006).

Metabolism of mycotoxins consists of biochemical modification of compounds, whereby lipophilic chemical compounds are converted into more readily excreted hydrophilic products, in two phases. These include: 1) phase I where a variety of enzymes act to introduce reactive and polar groups into their substrates by transformations such as reduction, oxidation and hydrolysis; 2) phase II or conjugation where modified phase I compounds are then conjugated by glucosidation, glucuronidation or sulfatation (Rubert et al., 2014). Hence, several biomarkers of mycotoxins may be found as conjugates of glucuronides, in biological samples.

Mycotoxins have been reported in breastmilk samples and food systems from many countries, globally (Coppa et al., 2019; Fakhri et al., 2019; Flores-Flores et al., 2015). Mycotoxin exposure has been implicated as a potential risk factor for growth faltering and stunting among children (Smith et al., 2012; Smith et al., 2015). It has been asserted that "*from a human-rights, food justice and public health point of view, aflatoxin contamination is a crisis and exposure is an expression of poverty*", according to ICRISAT scientist, Michael Hauser (Jerving, 2020).

2.2.1 Food systems, prevention and mitigation

1) Aflatoxins

Aflatoxins are difuranocoumarin derivatives produced by polyketide pathway of *Aspergillus flavus, parasiticus* and *nomius* strains (Goto et al., 1996). Twenty different AFs have been identified, of which AFB_1, B_2, G_1, G_2 have been found in foods and feed. AFB_1 is the most toxic and is classified as a group 1 carcinogen by the International Agency for Research in Cancer (IARC, 1993). Aflatoxins are commonly found in cereals such as maize, groundnuts and oil seeds which are contaminated with *Aspergillus flavus* and *Aspergillus parasiticus* (Shirima et al., 2013; Wild & Gong, 2010). Aflatoxin production is exacerbated by dry and hot climatic conditions and in pre-harvest crops due to drought, floods, delayed harvest, pest infestation, poor postharvest practices such as inadequate drying, handling and storage (Wild & Gong, 2010). Optimal temperatures for growth of aflatoxins range between 30-37 ºC with > 85% humidity.

AFB_1, when ingested by mammals is hydroxylated in the liver to 4-hydroxylated AFM_1 which is excreted in the milk of dairy animals and humans, AFM_2 is the hepatic hydroxylation byproduct of AFB_2 (Raiola et al., 2015).

Several strategies have been tested to reduce aflatoxin exposure at various levels of the food chain. Broadly, control of mycotoxins can be attained by: 1) preventing mold and fungus growth in crops and feedstuffs; 2) decontamination of mycotoxin contaminated feed and food; 3) continuous surveillance of mycotoxins in crops, feedstuffs and human food (Tola et al., 2016). The utilization of good agricultural practices is essential for prevention and containment of all mycotoxins. Early harvesting lowers the risk of aflatoxin development, in addition to proper storage and drying of crops which are effective tools to reduce mycotoxin production (Turner et al., 2005). Decontamination strategies include physical approaches such as sorting, washing and

crushing in addition to de-hulling maize and other grains to remove aflatoxins and fumonisins (Fandohan et al., 2005). Chemical approaches may include use of fungicides such as prochloraz, propiconazole, epoxiconazole, tebuconazole, cyproconazole, Oltipraz, chlorphylin and azoxystrobin (Haidukowski et al., 2005; Ni & Streett, 2005). Biological approaches involve use of non-toxic strains of the toxin producing molds, proper harvesting, storage and processing of agricultural crops and feedstuffs. Aflasafe, a novel atoxigenic strain of *A.flavus* L-morphotype has been tested and is used in some African countries as a biocontrol agent to competitively displace aflatoxin-producing isolates in the field, allowing production of crops with little to no aflatoxin contents (Cotty et al., 2006; Senghor et al., 2020).

Novel studies have tested the use of calcium montmorillonite clay to prevent illness associated with acute aflatoxin exposure. The inclusion of this clay in diet can lead to adsorption of aflatoxins tightly in the gastrointestinal tract and reduce its bioavailability. Awuor et al., (2017) have found the clay to be effective at reducing aflatoxin bioavailability in Kenya.

2) Ochratoxins

Ochratoxins are produced by *Aspergillus ochraceus* and *Penicillium verrucosum* strains of fungi. There are four primary forms of ochratoxins, namely A, B (dechlorinated OTA), OTC (ethylated OTA) and OTα. All of these compounds are known hepatotoxins, nephrotoxins, immunotoxins and teratogenic to animals (Raiola et al., 2015). OTA is the main ochratoxin of toxicological significance and is commonly found in cereals, animal source products such as cheese and meats, dry foods, beans and nuts (Aish et al., 2004). OTA is classified as a group 2B carcinogen by the IARC, or possibly carcinogenic to humans (Pfohl-Leszkowicz et al., 2007). Its production is impacted by climate, storage, transport conditions, milling, roasting and processing (Pitt, 2000). OTA is produced at temperatures ranging between 0-37ºC (Wu et al., 2014).

OTA has limited solubility in water and binds strongly to plasma proteins. It becomes reabsorbed in the kidneys and into enterohepatic circulation from the gastrointestinal tract, therefore biotransformation or renal clearance is delayed, and the half-life of OTA is approximately 35 days (Ringot et al., 2006; Studer-Rohr et al., 2000). OTA has been found in maternal and fetal serum and is known to cross the placental barrier, although mechanisms have not been fully elucidated (Zimmerli & Dick, 1995; Postulpolski et al., 2006).

In milk, excretion of OTA is supported by breast cancer-related protein, which is a member of the ATP-dependent efflux transporter family, also known to be responsible for excretion of other xenobiotics (Jonker et al., 2005; Schrickx et al., 2005; EFSA, 2006; Gurbay et al., 2009). Some studies suggest that OTA analogs are cleared from the body at much faster rates than OTA itself (Clark and Snedeker, 2006). Therefore, methods that could enhance the formation of OTA metabolites may result in lowered toxicity.

Several different strategies have been developed to contain OTA contamination, including use of microorganisms such as actinobacteria, bacteria, filamentous fungi, yeast that have the ability to degrade and/or adsorb OTA. Biological degradation of OTA to non-toxic OTα is another effective strategy (Chen et al., 2018). Microorganisms with good OTA degradation and adsorption ability in addition to OTA degradation enzymes have been applied in the food and feed processing industries (Chen et al., 2018).

3) Fumonisins

Fumonisins are produced by *Fusarium verticillioides, F. proliferatum and A. niger* fungi (JECFA, 2011). *F. verticilioides* is found in maize, globally and can cause Fusarium ear rot under certain climatic and environmental conditions (Wu et al., 2014). FB_1 is the predominant fumonisin metabolite (70%) and found in commodities including maize, corn, rice, sorghum,

pearl millet, beans, wheat, chilies and soy beans (Deepa and Sreenivasa, 2017). FB_1 is a group 2B or potential carcinogen for humans according to IARC classification (IARC, 1993).

4) Deoxynivalenol

Deoxynivalenol is a trichothecene that belongs to secondary metabolites produced by *Fusarium, Myrothecium and Stachybotrys* during the process of food growth (Grove, 2000). Environmental conditions that favor growth of 4-deoxynivalenol accumulation in food crops include nitrogen fertilizers, minimal tillage, application of azoxystrobin (fungicide) or glyphosate (herbicide) and through cultivation of grains where maize has been grown in previous years (Raiola et al., 2015). DON is found predominantly in cereals including wheat, maize, barley and less often in rice, oats, rye, triticale and sorghum. DON is recognized as a group 3 carcinogen by IARC, and its carcinogenicity is not classifiable for humans (IARC, 1993).

2.2.2 Interactions between multiple mycotoxins

Mycotoxins are generally resistant to industrial food processes such as milling, processing, and heating and are therefore able to persist in the food and feed supply chain unabated (Bullerman et al., 2007). Due to their known co-occurrence in food and feed, it is important to understand the effects of multiple mycotoxins on human health (Kifer et al., 2020).

Interactions may occur between concomitantly occurring mycotoxins which can be *antagonistic, additive or synergistic* in nature (Grenier et al., 2011). *Synergistic interactions* can be categorized as follows: 1) Effects of mycotoxins combined are greater than expected from the sum of the individual effects of the two toxins. Potentiation occurs when one toxin does not display any effect but the effect of co-contamination is greater than the effect of the other toxin alone; 2) The second type of synergism occurs when two mycotoxins induce opposite effects and the combined treatment induces an effect that is greater than the individual effect. Here, although

the toxins alone have opposite effects, their interaction leads to an exacerbated effect; 3) two mycotoxins induce similar effects and their combined effects induce an opposite effect than the individual effect.

Additive effects include those where the combination effect of two or more mycotoxins can be calculated as the sum of their individual effects. A 'less than additive' effect is described as one where the effect of combined treatment reflects the effect of only one of the toxins without the additional effect of the other toxin. Finally, *antagonistic effects* can be described as those where two mycotoxins induce similar effects and their combined treatment induces a lower effect. The combination, therefore lowers the effect of the more potent toxin. Antagonistic effects can also occur when two mycotoxins do not induce the same effects and the combined treatment induces an effect that is in between the two effects. The combination lowers the effect of one of the toxins (Grenier et al., 2011). Limited studies exist on the toxicological effects of mycotoxins, and most have been conducted on laboratory or farm animals.

Associations between AFs and FBs have been shown to induce a significant increase in serum levels of aspartate transaminase, alkaline phosphatase, alanine transaminase and gamma-glutamyl transferase in synergistic and additive interactions (Grenier et al., 2011). Interactions between AFs and OTA have been reported to be less than additive or antagonistic (Kalorey et al., 2005). AFs and OTA are able to cross the placental barrier and have been shown to have teratogenic properties in some species (Wangikar et al., 2004b; Wangikar et al., 2005). Similarly, less than additive and antagonistic interactions have been observed between DON and AFs (Huff et al., 1986). As mycotoxins generally co-occur in food items, further research on the presence of multiple toxins in breastmilk and other biological samples is warranted, in addition to their interactions and associations with health outcomes (Han et al., 2014; Grenier & Oswald, 2011).

2.2.3 Regulatory limits

Regulatory limits are set for mycotoxins of public health importance by national and international regulatory agencies responsible for food safety (FAO). Toxicological data and data on the occurrence of mycotoxins in various food commodities are used to inform **hazard assessment** and **exposure assessment**. Regulations are made based on known toxic effects. Hazard and exposure assessments in turn inform **risk assessment**, which is the scientific evaluation of the probability of occurrence of known or potential adverse health effects resulting from human exposure to food-borne hazards, and is the primary scientific basis upon which regulations are established (Van Egmond & Jonker, FAO, 2002). Mycotoxins of primary importance to public health, namely, aflatoxins, ochratoxins, fumonisins, deoxynivalenol and zearalenone are regulated (Van Egmond & Jonker, 2003).

Aflatoxins are regulated by the Codex Alimentarius Commission, in addition to various national and international regulatory bodies. The US Food and Drug Administration (FDA) has established maximum allowable levels of total aflatoxins in food items at 20 ppb. European Commission (EC) legislation places maximum levels of AFB_1 in cereals at 2.0 ppb with total AFs ($B_1 + B_2 + G_1 + G_2$) at 4.0 ppb. In milk, AFM_1 must not exceed 0.05 ppb. Additional regulations on levels in infant formula and follow-on formula are capped at 0.025 ppb. Food Safety & Standards Authority of India regulates aflatoxins in cereals, cereal based products, nuts, pulses, and oilseeds at levels of 15 ppb and AFM_1 in milk at 0.5 ppb. Maximum permissible limits for total AFs in all food commodities for sale on the Indian market are 30 ppb (Sharma et al., 2017). Indian laws allow for AF levels in food items and milk that are 2-10 times higher than those sold on EU markets, thus making domestic regulation less stringent than levels set by other international regulatory agencies.

OTA is regulated in wheat, barley and rye at 20 µg/L by the Food Safety and Standard Authority of India (FSSAI). Codex Alimentarius standards for OTA in unprocessed wheat, barley and rye are set at 5 µg/L, European Union limits are set at 5 µg/L for unprocessed cereals and 3 µg/L for cereals intended for direct human consumption (Eskola et al., 2019). The CODEX Alimentarius commission has set Fumonisin B_1 + B_2 levels in unprocessed maize at 4000 µg/kg (Eskola et al., 2019). FSSAI maximum limits for DON in wheat are set at 1000 µg/kg. A comparison of regulatory limits set by JECFA/FAO, USFDA, EC and FSSAI for mycotoxins in food items is provided in **Table 1**, below:

Table 1: Summary of maximum tolerable levels[a] for mycotoxins in assorted food items set by global regulatory agencies

Food Items	Mycotoxin	Regulatory Body			
		JECFA/FAO-Codex Alimentarius	FSSA India	EU Commission	US FDA
All food products	$AFB_1+B_2+G_1+G_2$ & AFM_1		30 µg/kg	4 µg/kg	20 µg/kg
Animal feed (peanut meal)	AFB_1		120 µg/kg	5 µg/kg	20 µg/kg
Cereals	AFB_1			2 µg/kg	
	Total AFs		15 µg/kg	4 µg/kg	
Milk	AFM_1	0.5 µg/kg	0.5 µg/L	0.05 µg/kg	0.5 µg/L
Maize and rice	AFB_1			5 µg/kg	20 µg/kg
	$AFB_1+B_2+G_1+G_2$			10 µg/kg	
Wheat, barley, rye	OTA	5 µg/L	20 µg/L		
Cereals	OTA			3 µg/L	
Unprocessed cereals	OTA			5 µg/L	
Maize	$FB_1 + FB_2$	4000 µg/kg			
Wheat, maize, barley flour	DON	1000 µg/kg	1000 µg/kg	750 µg/kg	

[a]Maximum tolerable level for a contaminant in food or feed commodity is the max concentration of that substance recommended by the Codex Alimentarius Commission (CAC) to be legally permitted
[b]CODEX Standard 1995 (http://www.fao.org/fileadmin/user_upload/livestockgov/documents/1_CXS_193e.pdf)
[c]Petersen, 2018 (https://tinyurl.com/yaebcktx)
[d]WHO, 2018 (http://www.inchem.org/documents/jecfa/jecmono/v74je01.pdf)
[e]FAO, 2003 (http://www.fao.org/3/a-y5499e.pdf)
[f]van Egmond & Jonker, 2004 (https://www.jstage.jst.go.jp/article/myco1975/2003/Suppl3/2003_Suppl3_1/_pdf)
[g]Mahato et al., 2019 (https://www.frontiersin.org/articles/10.3389/fmicb.2019.02266/full)

Regulations have been adopted by several domestic and international food safety bodies to control levels of toxic compounds in foods intended for infants and young children. The European Union has set limits for various mycotoxins in infant formula and follow-on formula shown in **Table 2** (van Egmond & Schothorst, 2007).

Table 2: Maximum limits for aflatoxins in infant formula and foods set by the FAO/WHO, EC and USFDA

Mycotoxin	**Food product**	**Regulatory Agency Limit (µg/kg)**		
		EC	**FAO/WHO**	**US FDA**
AFM_1		0.025	0.05 (milk)	0.5 (milk)
AFB_1	Cereal based foods and infant foods, infant formula, follow-on formula, dietary foods for special medical purposes intended for infants, processed cereal-based foods and baby foods for infants and young children	0.1	5 (milk producing animals)	-
FBs (B_1 + B_2)		200		
OTA		0.5		
DON		200		
Patulin		10		
Zearalenone		20		

[a]EC = European Commission; FAO/WHO = Food & Agricultural Organization/World Health Organization; US FDA = United States Food and Drug Administration

Health Canada has set limits for OTA in infant foods and DON in uncleaned wheat ingredients used in such products at 500 ng/kg and 1,000,000 ng/kg respectively (Health Canada, 2012).

2.2.4 Hazard identification & characterization and Exposure assessment

Hazard Assessment

Qualitative indications that a contaminant can cause adverse effects on health (hazard identification) are used by the Joint Committee for Food Additives (JECFA/FAO) to establish the nature of adverse effects (hazard characterization). Together, hazard identification and characterization establish the conditions by which a certain mycotoxin has the potential to cause adverse health effects or disease and the quantitative relationship between level of dietary exposure (dose) and associated adverse effect (response) (Assuncao, Silva & Alvito, 2016). This evaluation of toxicological data results in establishment of '**provisional tolerable weekly intake' (PTWI)** or **'provisional tolerable daily intake' (PTDI)** values. Here, provisional indicates the tentative nature of such evaluations, given that reliable data on the consequences of human exposure at levels of concern may not be available to JECFA. **Tolerable daily intakes** (TDIs) represent tolerable intakes that are expressed on a daily or weekly basis (TWIs), whereas PTDIs and PTWIs, are used for contaminants that may accumulate in the body (Herrman & Younes, 1999).

Evaluations are based on determination of a no-observed-adverse-effect-level (NOAEL) in toxicological studies and application of an uncertainty factor (Van Egmond & Jonker, 2003). Exposure assessments (presented in chapter 4) involve estimating the frequency, intensity and duration of ingestion of a mycotoxin, by combining food consumption data with data regarding the occurrence of chemical substances in a number of food items (Assuncao et al., 2016). Risk characterization (as presented in chapter 4), integrates the results of exposure assessment with those of hazard characterization to estimate the degree of concern (Assuncao et al., 2016).

As AFs are carcinogenic and genotoxic, no maximum tolerable daily intake levels have been established, although Kuiper-Goodman (1998) has recommended a PMTDI value of 1 ng kg^{-1} bw day^{-1}. Limits for AFs in food items should be **as low as reasonably possible (ALARA)**. The ALARA level is defined as the concentration of a substance that cannot be eliminated from a food without involving discarding the food altogether or without severely compromising the availability of major food supplies (Van Egmond & Jonker, 2003). Maximum tolerable weekly intake values (PMTWI) for OTA are 120 ng kg^{-1} bw day^{-1} (van Egmond & Jonker, 2004). As FB_1 is nephrotoxic, PMTDI values are set at 2000 ng kg^{-1} bw day^{-1} (WHO, 2002), and PMTDI for DON is set at 1000 ng kg^{-1} bw day^{-1} (Canady et al., 2001). A detailed list of various PMTDI values is provided in **table 3** below.

Risk Characterization

Several different methods exist to characterize risk of toxic compounds, based on their genotoxic and carcinogenic potential (Assuncao et al., 2016). EFSA set **health-based guidance values (HBGV)** for substances present in foods for infants below 16 weeks of age have taken into consideration several factors including maturity of gastric, pancreatic and biliary functions; absorption of substances may be slower in infants and development of phase I and II metabolic enzyme isoforms (Hardy et al., 2017). **Hazard quotient (HQ)** can be derived by comparing reference dose (tolerable daily intake) with exposure to evaluate whether the exposure level is tolerable or not, where a ratio of $HQ < 1$ indicates tolerable exposure and a ratio of $HQ > 1$ indicates non-tolerable exposure levels (EFSA, 2013). A **hazard index (HI)** is defined as the sum of the respective HQs for individual mixture components, and is calculated as the ratio between exposure and reference dose (Borg et al., 2013; Assuncao et al., 2016).

For carcinogenic AFs, a **margin of exposure (MOE)** is calculated, as the ratio of Benchmark Dose Lower Confidence Limit ($BMDL_{10}$) and the level of PDI (exposure) (Benford et al., 2010). Values below 10,000 indicate a high public health concern (EFSA, 2013). Coppa et al., (2019) note that studies of AFM_1 in breastmilk and infant formula from many countries were below this threshold, thus indicating need for risk management. A combined MOE is called a **MOET**, and is used for mixtures of chemicals that are genotoxic and carcinogenic, calculated as the reciprocal of the sum of reciprocals of individual MOEs (Assuncao et al., 2018).

There is a need for systematic and large-scale studies of multiple mycotoxin contamination in infant foods and cereal based food consumed by infants and young children and the potential impact of combined exposure on the health of children, globally.

Table 3: Tolerable daily and weekly intake values for various mycotoxins

Mycotoxin	PMTDI	TDI for Infants[a]	PMTWI	Agency/Reference
AFB_1	1 ng/kg bw/day	-	-	Kuiper-Goodman (1998)
AFM_1	0.2 ng/kg bw/day	-	-	JECFA
OTA	100 ng/kg bw/day		0.01 mg/kg bw/week	JECFA
			120 ng/kg bw/week	EFSA
OTA in BM	14 ng/kg bw/day	-	100 ng/kg bw/week	Munoz et al., (2014)
	5 ng/kg bw/day	17.4 ng/kg bw/day		EC 1998 recommendation
	16 ng/kg bw/day			FAO/WHO, 2007
FB	2000 ng/kg bw/day	-	-	FAO/WHO 2011
DON	1000 ng/kg bw/day	1000 ng/kg bw/day	-	FAO/WHO, 2011

[a]Kuiper-Goodman (1998); Munoz et al., (2014)
[b]Provisional maximum tolerable daily/weekly intake (PMT(D/W)I); TDI = tolerable daily intake

Coppa et al., (2019) in their systematic review of breastmilk mycotoxin studies found that probable daily intake values for AFM_1 through breastmilk ranged between 0.003-917 ng kg^{-1} bw day^{-1}, accounting for variations in prevalence and concentrations of mycotoxins from different parts of the world. Probable Daily Intake (PDI) values were lower from consumption of infant formula. These values range from being under to several folds over PMTDI values described in **table 3**.

2.2.5 Health outcomes

Aflatoxin exposure has been associated with hepatocellular carcinomas, immune suppression (Jiang, et al., 2005), low birth weight (Shuaib et al., 2010), hepatomegaly (Gong et al., 2012) and acute mycotoxicosis (Probst et al., 2014; Williams et al., 2004). AFB_1 exerts it's toxic effects by reducing anti-inflammatory cytokine IL-4 expression, increasing pro-inflammatory cytokine IFN-g and TNF-α expression by NK cells, and repeated exposure facilitates inflammatory responses by regulation of cytokine expression (Mehrzad et al., 2014). AFs can reduce the efficiency of immunization in children, likely driven by these mechanisms (Raiola et al., 2015). Aflatoxins also exert synergistic effects with hepatitis B virus resulting in hepatocellular carcinoma by integration of the hepatitis B virus x gene, interference with nucleotide excision repair, alterations in methylation of genes and DNA mutations (Kew, 2013). In humans, AF metabolism occurs via microsomal cytochrome P450 monooxygenases which are found in all tissue but are more active and higher in concentration in the liver (Hussein et al., 2001). Children are particularly vulnerable to the risk of cancer due to AFs and repeated exposure in-utero and throughout childhood can predispose them to risk of liver cancer later in life.

Ochratoxin A has been implicated as a cause of urinary tumors in infants and young children (Raiola et al., 2015), and has also been associated with Balkan endemic nephropathy

(Roupret et al., 2015). The adverse effects of OTA may be exerted by mechanisms that include genotoxicity (Pfohl-Leszkowicz et al., 2012), histone acetyltransferase inhibition (Czakai et al., 2011) and cytoskeleton destabilization (Rached et al., 2006). OTA also inhibits nuclear factor erythroid 2-like (Nrf2), which represents major oxidative stress response and regulates transcription of genes involved in reduction of reactive oxygen species, quinones and glutathione synthesis (Jennings et al., 2012).

In addition to OTA's adverse effects on the kidney, studies have also shown OTA mediated changes to the gut leading to altered nutrient absorption in the intestine (Liew & Redzwan, 2018). In vitro studies have shown a decrease in glucose absorption via SGLT1 transporters in the presence of OTA, in addition to increased intestinal permeability (Maresca et al., 2010). OTA treated animals also experience faster and more harmful parasite infections, in addition to higher lesion and oocyst indices and more damage to the mucosa of the intestines (Manafi et al., 2011). OTA induced oxidative stress can alter intestinal permeability (Anderson et al., 2016). Finally, inflammatory pathways in the intestine are also affected by OTA. In piglets treated with OTA, inflammatory cytokines including IL-8, IL-6, IL-17A, IL-12 and IL-18, were all seen in significantly lower concentrations (Marin et al., 2017). Alterations to the immune response can increase vulnerability to infections in the gut. Nutrient absorption, disrupted intestinal permeability, cell apoptosis and modulation of immune response are all known effects of OTA exposure.

Fumonisins exert toxic effects through inhibition of sphingolipid metabolism and ceramide synthase which result in accumulation of sphingosine in cells (Masching et al., 2016). Normal degradation of sphingolipids to ceramides require sphingomyelinase and ceramidase (Boini et al., 2017). Sphingosine accumulation has been linked to neurological and

immunological diseases such as cancer (Boini et al., 2017). In the gut, FB_1 accumulation leads to growth inhibition and apoptosis of intestinal epithelial cells (Angius et al., 2015). FB_1 is also known to alter the integrity of the intestinal barrier by suppressing the expression of tight junction protein (Romero et al., 2016). Increased intestinal permeability leads to translocation of bacteria and other pathogens (Kelly et al., 2015).

Fumonisins have been implicated as a risk factor for esophageal cancer in humans (Wu et al., 2014). Due to their effects on sphingolipid metabolism and folate transport across cell membranes, FBs have also been shown to induce neural tube defects in mice (Gelineau-van Waes et al., 2005). Populations in which folate consumption is low and maize consumption is high, and where climate and environment favor fumonisin accumulation, may be particularly susceptible to the NTDs (Wu et al., 2014). Fumonisin exposure can be estimated by the ratio of serum sphinganine:sphingosine (Sa/So). Although this biomarker has not been validated in humans, studies have shown an increased dose-response relationship between maternal Sa/So and adjusted ORs for NTDs in babies (Wu et al., 2014).

Deoxynivalenol exerts effects on the immune system by inhibiting protein biosynthesis and altering production of pro-inflammatory cytokines (Desjardins, 2006; Awad et al., 2013). Additionally, DON can also bind to peptidyl transferase and inhibit the synthesis of RNA and DNA, while modifying cell membranes (Feinberg and Mclaughlin, 1989). DON can also interfere with the production of TNF-α, a pro-inflammatory cytokine involved in systemic inflammation and an acute phase protein, which can in turn impact susceptibility to infectious diseases, and has been associated with the growth hormone system (Raiola et al., 2015; Pestka et al., 2010). Exposure to DON has also been associated with impaired intestinal immunity,

diarrhea, and vomiting, thus leading to its alternate name, vomitoxin (Lombard 2014; Smith et al., 2012).

2.2.6 Association with pregnancy outcomes

Epidemiological evidence from several countries across the globe has shown that mycotoxin exposure is widespread among pregnant women and newborns (Abdulrazzaq et al., 2002; Chan-Hon-Tong et al., 2013; Groopman et al., 2014; Jonsyn et al., 1995). Smith et al., (2017) have suggested that chronic AF exposure could contribute to anemia through mechanisms related to immune activation and enteropathy, decreased capacity of the intestine to absorb essential nutrients such as iron, a decrease in erythropoiesis due to chronic inflammation and reduced availability of iron due to hepcidin upregulation. This in addition to zinc deficiency, pre-pregnancy weight gain, maternal body mass index, height and other environmental and socioeconomic indicators may mediate the relationship between AF exposure in pregnancy and adverse outcomes including low birth weight (Smith et al., 2017).

Mechanisms whereby mycotoxins can affect pregnancy outcomes include multiple pathways in both mothers and children and include: 1) upregulation of pro-inflammatory cytokines and/or downregulation of anti-inflammatory cytokines; 2) enteropathy characterized by intestinal inflammation and impaired placental development; 3) toxic effects on fetal organs resulting in inflammation and impaired fetal development (Smith et al., 2017; Kyei et al., 2019).

Maternal Outcomes

A study conducted in Ghana found that mothers with higher AF exposure had 35% higher odds of stillbirth (aOR = 1.35; 95% CI: 0.52-3.50) (Shuaib et al., 2010). Norwegian studies have also shown that mycotoxin exposure is associated with increased mid-pregnancy deliveries (gestational age 21-24 weeks; OR = 1.58; 95% CI: 1.19-2.09), and late-term miscarriages

(gestational age 16-27 weeks, OR = 1.31; 95% CI: 1.11-1.55). No associations were found with perinatal death (OR = 1.05; 95% CI: 0.97-1.13) (Kristensen et al., 1997; 2000; Nordby et al., 2006). Moodley et al., (2001), found maternal blood FB levels to be highest in eclamptic pregnant women (2.85 ± 0.08 µg/mL) > pre-eclamptic women (0.45 ± 0.17 µg/mL) > normotensive women (0.32 ± 0.08 µg/mL).

Child Outcomes

Abdulrazzaq et al., (2002, 2004) have found that AF exposure is negatively associated with mean birth weight (r = -0.63 and -0.57). Other studies have also found AF exposure to be associated with birth weights between 34-70 g for infants who had detectable aflatoxins or mothers with higher AF exposure (Abulu et al., 1998; Maxwell et al., 1994; Turner et al., 2007).

Jonsyn et al., (1995), did not find statistically significant associations between birth weight and exposure to AFs and OTA, although they did note that the mean birth weight of exposed female children in their study, was lower than unexposed girls. Positive associations have been documented between low birth weight (OR_{range} = 1.07-2.29), and AF exposure across several studies (Abdulrazzaq et al., 2004; Carlos et al., 2014; Jonsyn et al., 1995; Lauer et al., 2018; Shuaib et al., 2010c). AF exposure has also been linked to neonatal jaundice, where studies have found the odds of AF exposure to be 2-fold (aOR: 2.68; 95% CI: 1.18-6.10), in jaundiced neonates compared to non-jaundiced neonates (Abulu et al., 1998; Sodeinde et al., 1995). These associations may be explained by the toxic effects of AFs on newborn liver, coupled with increased hemolysis of fetal hemoglobin (Kyei et al., 2019).

In Mexico, a study looking at aflatoxin exposure found positive associations between maternal consumption of contaminated foods and neonatal death (OR = 2.35; 95% CI: 1.43-3.86) (Carlos et al., 2014). More recently, Lauer et al., (2018) have also found associations between

maternal aflatoxin exposure (AFB_1-lysine adducts) during pregnancy and adverse birth outcomes. Elevated levels of maternal AFB-Lys levels were significantly associated with infant outcomes at birth including lower weight (adjusted β: -0.07, 95% CI: -0.13, -0.003; p = 0.040), lower weight-for-age z-score (adjusted β: -0.26, 95% CI: -0.49, -0.02; p = 0.035) and lower head circumference-for-age z-score (adjusted β: -0.23, 05% CI: -0.43, -0.03; p = 0.023) (Lauer et al., 2018). In the AflaCohort Study, Andrews-Trevino et al., (2019), followed children pre-partum (< 30 weeks gestation) through to delivery, to measure birth outcomes, and found relatively low maternal serum AFB1-lysine adduct concentrations (geometric mean 1.37 pg/mL, 95% CI: 1.30, 1.44 pg/mL) to be significantly associated with small-for-gestational age infants (aOR: 1.13, 95% CI: 1, 1.27, $p < 0.05$), but not low birth weight, stunting or pre-term birth (Andrews-Trevino et al., 2019).

Maternal exposure to Fumonisins and associations with birth outcomes have been examined in far fewer studies, of which Missmer et al., (2006) found maternal serum sphinganine:sphingosine (Sa:So) ratios, to be associated with increased risk of neural tube defects (OR_{range} = 1.5-4.5), up to a threshold ratio of 0.35. Beyond this Sa:So ratio, the risk of fetal death was increased. Strong bodies of evidence link FB with disruptions in biosynthesis of sphingolipids, which are vital structures in cell membranes. Interference in folate receptors and folate bioavailability due to FB exposure leads to neural tube defects (Gelineau-van Waes et al., 2012; Marasas et al., 2004).

2.2.7 Breastmilk and infant foods

Breastmilk is a potential source of mycotoxin exposure for infants and young children, where contaminants may become excreted by way of maternal diet. Breast cancer resistance protein is likely involved in mechanisms of mycotoxin excretion into breastmilk (Jonker et al.,

2005), and this protein is a part of the ATP-dependent efflux transporter family (Van Herwaarden et al., 2006). Lower pH and higher lipid concentrations in milk compared to plasma can result in the excretion of toxic compounds into breastmilk (Ito et al., 2003). Upon excretion, mycotoxins can be partitioned by their physico-chemical properties in different phases of milk, where for instance AFM_1 is found predominantly in the skim milk portion associated with casein due to hydrophobic interactions (Campagnollo et al., 2016).

AFM_1 and OTA are the main mycotoxins that have been found and studied in human milk samples. Few studies have also documented the presence of unmetabolized AFB_1, AFB_2, AFG_1 and AFG_2 from countries including Egypt (Polychronaki et al. 2007), Italy (Galvano et al., 2008), Turkey (Gurbay et al., 2010) and Brazil (Andrade et al., 2013). AFM_1 is detected in breastmilk between 12-24 hours after AFB_1-consumption, with levels decreasing rapidly over time once AFB_1 intake is stopped. The toxin is undetectable 3 days after withdrawal (Battacone et al., 2009). Between 0.09-0.43% of AFB_1 can be excreted as AFM_1 in milk (Mahdavi et al., 2010; Zarba et al., 1992). Carry-over rates for OTA from maternal diet into breastmilk are not determined (Munoz et al., 2014).

Maternal dietary patterns including the consumption of dairy products such as milk in Iran and breads, bakery products and cured pork meats in Italy, have been associated with the presence of AFM_1 and OTA in breastmilk samples (Mahdavi et al., 2010; Galvano et al., 2008). Additional determinants of exposure include socioeconomic status and seasonality (Cherkani-Hassani et al., 2016). Systematic reviews and meta-analyses of breastmilk mycotoxins have been conducted and demonstrated the presence of AFM_1 in colostrum and breastmilk (Coppa et al., 2019) from across the globe. Levels of AFM_1 were particularly high from studies conducted in Egypt (200-19,000 ng/L), Sudan (7-2561 ng/L), Nigeria (1-601 ng/L) and Iraq (100-3010 ng/L)

(Tomerak et al., 2011; Elzupir et al., 2012; Anthony et al., 2016; Qadir et al., 2014). Up to 100% of study samples have been found to be positive for mycotoxins and associations are highly variable across studies dependent on maternal dietary habits in different countries.

OTA has been detected at high frequencies (30-100%) of samples in studies from Brazil, Iran, Italy and Slovakia at concentrations ranging between 1-75 ng/L (Coppa et al., 2019). In Chile, OTA in breastmilk has been detected at concentrations up to 186 ng/L (Munoz et al., 2010; 2014a), and between 110-7340 ng/L in Iran and 621-13,111 ng/L in Turkey (Kamali et al., 2017; Gurbay et al., 2009), respectively. In Italy, Galvano et al., (2008), found OTA in breastmilk at concentrations ranging between 5-405 ng/L. In Sierra Leone, OTA has been found at concentrations between 200-337,000 ng/L in 35% of breastmilk samples (Jonsyn et al., 1995).

Few studies have quantified other mycotoxins such as FB_1 in breastmilk samples at concentrations ranging between 6-471 µg/L (Magoha et al., 2014a). ZEN, another commonly occurring mycotoxin has been detected in breastmilk samples from Spain at concentrations ranging between 2-14 µg/L, in addition to other mycotoxins occurring at low concentrations including fusariotoxins such as nivalenol, HT-2 and enniatins (A, A1, B1, B2) (Rubert et al., 2014). *To the best of our knowledge, no studies have quantified mycotoxin concentrations in breastmilk samples from India.*

In addition to breastmilk, mycotoxins may also be found in infant formula and weaning foods. Although the WHO recommends that infants under 6 months of age be exclusively breastfed, these recommendations cannot be followed by many women due to extenuating circumstances, in which case the use of breastmilk substitutes such as formula become imperative (Boue et al., 2017). Several different mycotoxins have been detected in infant formula

products from countries including Burkina Faso (AFB_1: ~ 87,400 ng/kg with 84% of samples > LOQ; OTA: ~ 3200 ng/kg; $FB_1 + FB_2$: ~ 672,900 ng/kg) (Ware et al., 2017).

AFB_1, AFM_1, OTA are the primary mycotoxins detected in infant formula, and studies from countries including Brazil, Canada, Portugal among others have detected these in infant formula samples at levels ranging from 0.003 µg/kg to 0.886 µg/kg (Khaneghah et al., 2019). Studies conducted on infant milk food, formula and milk based cereal weaning foods, in addition to liquid milk samples have shown AFM_1 contamination in the range of 65-1012 ng/L in milk products and 28-164 ng/L in liquid milk from India (Rastogi et al., 2004). A study from the Indian state of Goa found that 100% of infant formula samples exceeded EC limits of 50 ng/L for AFM_1 and 75% of samples exceeded FSSAI limits of 500 ng/L for AFM_1 (Kanungo & Bhand, 2014), AFM_1 was found at lower concentrations in infant milk formula (501-713 ng/L) compared to liquid milk (511-809 ng/L) (Kanungo et al., 2014). More recently, Gummadidala et al., (2019) found 100% of infant food samples positive for AFM_1 and exceeding 25 ng/kg EU guideline levels. DON was found in 66% of infant food samples collected from Kolkata, India, with 6.9% of samples exceeding EU guidelines for baby food products (200 µg/kg) and 51.7% of samples with DON levels where dietary intakes could exceed 1 µg/kg recommendations by JECFA on food additives. AFs were not detected in any of these samples.

2.2.8 Associations with child growth outcomes

Evidence for the effects of mycotoxins on human growth is limited, however animal studies show that chronic exposure to AFs retards growth and can interfere with micronutrient absorption and utilization (Smith et al., 2012). Mechanisms whereby AFs impact growth include: 1) gastrointestinal cytotoxicity leading to increased diarrhea and abdominal pain, enterocyte damage leading to systemic immune activation; 2) interference with carbohydrate metabolism,

inhibition of protein synthesis leading to impaired metabolism and synthesis of fatty acids and phospholipids; 3) exacerbation of diseases known to result in growth retardation; 4) interference with availability and utilization of nutrients and zinc deficiency (Bennett & Klich, 2003). AF exposure is also an important proxy for poor diet quality.

FUM (FB) exposure may result in growth retardation and stunting because of their ability to increase cell permeability and disrupt sphingolipid metabolism by inhibiting ceramide synthase (Smith et al., 2012). Sphingolipids are essential to cell membrane integrity and disturbances in this biosynthetic pathway can affect intestinal epithelial cell viability and proliferation, modify cytokine production and modulate intestinal barrier function (Smith et al., 2012).

DON intake has been shown to induce a decrease in circulating levels of insulin like growth factor (IGF-1) in mice (Amuzie & Pestka, 2010). IGF-1 is an important mediator of growth hormone axis and hepatic IGF acid-labile subunit (IGF-ALS). Protein synthesis inhibition due to DON also leads to decreased circulation of IGF-1 and IGF-ALS, reduces the expression of claudin-4 which increases intestinal permeability and enables systemic immune activation. Additionally, the inhibition of SGLT1, GLUT5 and L-serine transporters can also cause diarrhea and increase intestinal permeability (Smith et al., 2012). AFs, FUM and DON may share convergent pathways leading to mucosal damage, impaired nutrient absorption and increased intestinal permeability and pathology that resembles EED and leads to eventual growth faltering and stunting.

OTA exerts it's toxic effects by blocking protein synthesis and energy production, the formation of DNA adducts, apoptosis and inducing oxidative stress (Koszegi & Poor, 2016). OTA can also alter nutrient absorption in the intestine, decreasing glucose absorption via SGLT-

1 transporters (Peraica et al., 2011). OTA fed animals experience faster and more harmful parasitic infections, have higher lesion and oocyte indices in the intestine and more damage at the mucosa, due to increased intestinal permeability (Manafi et al., 2011). Additional studies have shown that tight junction proteins, responsible for intestinal integrity are significantly ($p < 0.05$) suppressed and oxidative stress induced by OTA can alter intestinal permeability (McLaughlin et al., 2004). Inflammatory pathways in the intestine are also affected by OTA, with decreased expression ($p < 0.05$) of cytokines including IL-8, IL-6, IL-17A, IL-12, IL-18 (Marin et al., 2017), thus increasing risk of infections in the gut (Liew & Mohd-Redzwan, 2018).

Tesfamariam et al., (2019), in their systematic review of the literature on mycotoxins and growth outcomes note that the overall quality of evidence on the topic is poor, with inconsistent results on the associations between mycotoxin exposure and child growth indicators. They note that inconsistencies likely occur due to heterogeneity among studies in terms of matrices used (blood-plasma, serum, urine, breastmilk), measurement methods (HPLC, ELISA, LC-MS/MS), exposure period, seasonal variation, failure to adjust for confounders, differences in study populations and limitations with sample size.

To date, only two major randomized controlled trials have been conducted to examine the impacts of dietary mycotoxins on infant growth outcomes. A cluster RCT conducted in Tanzania, which sought to evaluate the effectiveness of locally available post-harvest mitigation strategies in preventing and reducing AF and FB contamination in maize ($n = 300$ children, 30 villages, 15 intervention and 15 control) found that AF intake is inversely associated with underweight ($\beta = -0.007$; 95% CI: -0.009 to -0.0004; $P = 0.039$) (Kamala et al., 2018). Kamala et al., (2018) in their randomized control trial also found that FUM intake is inversely associated

with underweight (β = -0.041; 95% CI: 0.067 to -0.014; P = 0.003). Children in this study were between 0-6 months of age.

Another RCT (n = 1230 pregnant women enrolled), conducted in Kenya aimed to decrease dietary AF exposure by swapping contaminated maize with safe maize, and encouraging households to purchase clean maize. At midline (children 11-19 mo), these authors found a significant effect on child linear growth, and the intervention increased LAZ by 0.16 (95% CI: -0.009 to 0.33, p = 0.032), while reducing the prevalence of stunting by 7 percentage points (95% CI: -0.125 to -0.007, p = 0.015) (Hoffman et al., 2018). Overall the intervention had no effect on child LAZ (mean endline LAZ = -1.64) or the prevalence of stunting at endline (children 24 mo) (Hoffman et al., 2018). The intervention did reduce ln serum AFB_1-lysine adduct concentrations (-0.273, 95% CI: -0.547, 0.001, p = 0.025) at endline, however.

Prospective cohort studies have examined associations between dietary AF exposure and child malnutrition, of which four have reported that AF exposure is negatively associated with HAZ among children 0-3 years of age (Gong et al., 2004 (16-37 mo.); Turner et al., 2007 (0-14 mo.); Magoha et al., 2014 (<6 mo.); Natamba, 2016 (1-12 mo.)). LeRoy et al., (2018) reported that low-dose AF exposure is associated with greater child linear growth among children 6-12 months of age. Magoha et al., (2014) reported a small and significant inverse association between AFM_1 and WAZ, and Natamba (2016) reported AF exposure was associated with decreased infant linear growth in HIV-positive pregnant women. However, five cohort studies reported that AF exposure is not significantly associated with child malnutrition (Shirima et al., 2015 (6-14 mo.); Magoha et al., 2016 (1-5 mo.); Mahfruz, 2017 (0-36 mo.); Mitchell et al., 2017 (0-36 mo.); Chen et al., 2018 (24-36 mo.)). Ages of children in these cohorts were between 0-36 months.

Turner et al., (2007) found a dose-response relationship between AF-albumin adducts, WAZ and HAZ in children. Gong et al., (2004) and Turner et al., (2007) in their observational studies reported that higher AF exposure is associated with a decrease in height. Increased levels of dietary AF exposure were also strongly related to lower levels of weight-for-age in infants.

Cross-sectional studies have found that AF levels are related to a decreased HAZ, of these two studies reported dose-dependent decreases in WAZ and HAZ among AF exposed children (Gong et al., 2002; Mahdavi et al., 2010 (9-120-day old infants); Shouman et al., 2012). In Benin and Togo, a study reported AF exposure to be positively related to underweight ($p = 0.047$) (Gong et al., 2002). A study from Kenya found that consumption of AF-contaminated flour was related to wasting in children (Okoth & Ohingo, 2004 (3-36 mo.)).

In addition, four cohort studies have reported FB exposure to be associated with child growth indicators (Kimanya et al., 2010; Shirima et al., 2015; Magoha et al., 2016; Chen et al., 2018). Two studies showed urinary markers of FB to be associated with an increase in stunting in children, with a mean difference of 1.8 cm reduction in growth among Tanzanian children in the highest urinary FB_1 quartile when compared to the lowest exposure quartile (Shirima et al., 2015). Kimanya et al., (2010) and Magoha et al., (2016) found that FB exposure was negatively associated with weight and length at 12 months and with impaired linear growth.

Studies from countries in Africa and South East Asia have reported AF exposure more frequently in serum and urine of children with kwashiorkor, compared to normal and marasmic controls (Coulter et al., 1986; De Vries et al., 1987; Ramjee et al., 1992; Hatem et al., 2005). Two case-control studies reported that AFs were detected at higher concentrations in children with kwashiorkor or marasmic-kwashiorkor when compared to control or marasmus group (Hendrickse et al., 1982; Tchana et al., 2010). Golden & Ramadath (1987) found that exposure to

AFs may be associated with kwashiorkor among children in low income undernourished communities. More recently, Tchana et al., (2010) found AFs in urine samples from 45.5% of children with kwashiorkor and marasmic kwashiorkor in Cameroon.

In the Philippines, an inverse and statistically significant correlation was found between AF exposure and mortality in children with acute respiratory infections (Quiepo, 1990). A case-control study in Nigeria found that presence of serum AF was a risk factor for neonatal jaundice (Sodeinde et al., 1995). In South Africa, children with AF-positive serum were shown to have significantly lower hemoglobin levels, longer duration of edema, increased number of infections and longer duration of hospital stays when compared to AF-negative group of kwashiorkor children (Adhikari et al., 1994). Additionally, two studies from the Gambia have shown that hepatitis B virus positive carrier have higher levels of AF adducts than those with negative status (Allen et al., 1992; Turner et al., 2000). Overall evidence on the associations between mycotoxin exposure and child malnutrition remains inconclusive, however efforts to reduce contamination and mitigate exposure are needed to improve overall population health, food security, economic and trade benefits of mycotoxin free food commodities (Smith et al., 2012).

2.3 Pesticides

Pesticides are widely used in agricultural and residential settings to help increase supply of crops, fruits and vegetables, but also to control vector borne diseases such as malaria and visceral leishmaniasis. Exposure to pesticides can occur through various routes including inhalation, ingestion and dermal contact. Additionally, there are several pathways of exposure including consumption, occupational exposure and handling of food that may be sprayed with pesticides during preparation. Many pesticides are known neurotoxicants and can cause acute toxicity at high doses, and exert less harmful effects at lower doses (Vrijheid et al., 2016).

Pesticides can be classified based on their chemical structure i.e. *organochlorines, organophosphates, pyrethroids* and *carbamates*. They may also be classified based on their target, namely insecticides, herbicides, fungicides, rodenticides, molluscicides, nematicides and acaricides. Pesticides can also be grouped based on their mode of action, for e.g. acetylcholinesterase inhibitors, calcium channel inhibitors etc (Collotta et al., 2013). Classifications by toxicity are defined by the World Health Organization, categorized on LD50 levels and the International Agency for Research on Cancer (IARC), based on carcinogenicity (Collotta et al., 2013).

The neurodevelopmental concerns posed by currently used and historical pesticides are well documented in the literature. Organochlorines (OCs), organophosphates (OPs), and pyrethroids are known to cause adverse cognitive, behavioral, sensory, and motor effects in children (Eskenazi et al., 2007; Eskenazi et al., 2008; Kostyniak et al., 1999; Longnecker et al., 2001; Rogan et al., 2005). Exposures to pesticides and other environmental contaminants can permanently affect the body structures of infants and young children leading to changes in physiology and metabolism, and predisposing individuals to development of serious chronic pathologies such as cardiovascular, metabolic, respiratory and neurodegenerative diseases, later in life (Vrijheid et al., 2016).

The Developmental Origins of Health and Disease (DOHaD) paradigm has postulated the potential implications of exposure to pesticides and their impact on the health of multiple generations. Several studies have demonstrated that developmental exposures to environmental chemicals such as DDT can lead to effects in F1 generation (direct offspring) of the exposed, but also in F2 generation (grandchildren) via germ cells, and apparent effects in F3 and F4 generations as well. (Schug et al., 2011; Skinner et al., 2010; Rutter et al., 1983; Skinner et al.,

2008; Skinner et al., 2011; Wolstenholme et al., 2012; Wolstenholme et al., 2011; Wolstenholme et al., 2011; Haugen et al., 2015).

Pesticide residues have been quantified in human milk, infant formula, baby foods and in various crops and vegetables, globally (Rice et al., 2000; Chen et al., 2014). Thus, infants can be exposed to these chemicals through direct ingestion of a variety of food sources.

2.3.1 Food systems, prevention and mitigation

1) Organochlorines

Insecticides, rodenticides, acaricides:	α-HCH, β-HCH, γ-HCH (lindane), Δ-HCH, Heptachlor, Aldrin, Heptachlor Epoxide, Methoxychlor, Trans-chlordane, cis-chlordane, Dieldrin, Endrin, β-endosulfan, Endosulfan Sulfate, p,p'-DDT, p,p'-DDE, p,p'-DDD, α-Endosulfan, Endrin Aldehyde, Endrin Ketone
Fungicides and herbicides:	Hexachlorobenzene, Alachlor, Metalochlor, Butachlor, Simazine, Atrazine

Organochlorine pesticides such as chlordane, DDT, aldrin, dieldrin, endrin, and hexachlorobenzene are among a list of persistent organic pollutants or chemicals that accumulate in the environment over a period of time. These chemicals have been used extensively in agriculture, globally and last for several decades in the environment (Cok et al., 2012). OCPs are broad spectrum toxicants and become sequestered in the lipid-tissue of humans where they accumulate due to age because of their long half-life (Haris et al., 2001). As these compounds are highly persistent, low in polarity, lipophilic and hydrophobic, they bio-magnify once they enter the food chain, particularly in lipid-rich tissues in humans, leading to adverse effects on health (Ayotte et al., 2003; Luzardo et al., 2013; Muller et al., 2017). These compounds are volatile, stable and have related chemical structures showing chlorine substituted aliphatic or aromatic rings (Jayaraj et al., 2016). These compounds share physicochemical characteristics such as persistence, bioaccumulation and toxicity. Their persistence in the environment, is due to

a half-life greater than 2 months in water and 6 months in soil sediment, these can range from moderate (half-life ~ 60 days) to high (half-life ~ 10-15 years) (Jayaraj et al., 2016).

Rapid population growth and an emphasis on achieving food grain sufficiency have resulted in the substantial use of OC pesticides by Indian farmers. Yadav et al., (2015) estimated that over 100,000 tons of DDTs have been used in India alone, for agriculture and in malaria eradication programs. Residues of OCPs including dichlorodiphenyltrichloroethane (DDT) have been transported throughout the global environment for decades and are found in soil, water, sediment, several years after application (Barlas et al., 2006; Singh, 2001). Their persistence, volatility and atmospheric distribution through long-range transportation have resulted in contamination of air, water, soil and food in places such as India (Singh et al., 2005; Aulakh et al., 2006; Bedi et al., 2015). OCs including DDTs and HCHs are manufactured, used and exported on a large scale in India (Ali et al., 2014). It is estimated that there are more than 125 basic producers of large to medium scale and more than 500 pesticide formulations in India (Abhilash & Singh, 2009). The residues of these POPs have an impact on the environment and ecosystem, due to which their production has been banned or restricted under the Stockholm Convention (2001). India faces gaps between legislation and implementation. Despite regulations, unauthorized and smuggled trade of POPs continues to be reported in the country (Link, 2006).

Studies have found residues of DDT (*p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDE and *p,p'*-TDE) in rice samples from the Indian state of Haryana at median values of 0.01 mg/kg, with 90% of tested samples exceeding detection limits but none over the maximum residue limits of 0.1 mg/kg set by CODEX (Toteja, Mukherjee, Singh, & Saxena, 2003). Bhanti & Taneja (2005), found DDT in winter vegetables including tomato, cauliflower, cabbage, raddish and brinjal at mean

concentrations of 5.47 µg/L. These authors also detected DDT summer vegetables including spinach, bottle gourd, cucumber, smooth gourd and pumpkin at mean concentrations of 2.82 µg/L. All samples were collected from the north Indian city of Agra. More recently, Chourasiya et al., (2015) found concentrations of a total of 20 OCPs in winter vegetables to range between 127.3, 217.9 and 222.4 ng/g in radish, radish leaves and cauliflower, respectively. For summer vegetables, they found Σ_{20}OCP concentrations to range between 97.4, 135.4 and 83.8 ng/g in brinjal, okra and smooth gourd, respectively. None of the samples in this study exceeded maximum residue levels for ΣDDT (Chourasiya, Khillare & Jyethi, 2015). Agarwal et al., (2015), have found high concentrations of α and β isomers of endosulfans in the Yamuna river in Northern India. High levels of γ- HCH and malathion have also been detected in the Ganga river that flows through parts of Northern India, in addition to methyl parathion, endosulfan and DDT in water from Bhagalpur, Bihar. Malathion has also been detected in okra (0.027-0.425 mg/kg), green leafy vegetables including spinach and cabbage (ND-0.272 mg/kg) from Lucknow, India (Srivastava et al., 2011). Based on these findings, pesticide residue quantification in the food supply chain in India warrant further investigation.

2) Organophosphates

Insecticides, acaricide, avicide, nematicide:	acephate, diazinon, monocrotophos, disulfoton, methyl paraxon, phosphomidon, malaoxon, methyl chlorpyrifos, fenitrothion, malathion, fenthion, ethyl parathion, ethion, phosalone
Herbicides, fungicides:	propazine, captafol

Organophosphate pesticides are derivatives of phosphorous organic esters and generally thiol or amide derivatives of thiophosphoric phosphinic, phosphonic, phosphoric acids with additional side chains of phenoxy, cyanide and thiocyanate groups (Kumar et al., 2016; O'Brien, 2016). These compounds are the main components of herbicides, insecticides and other pesticides and are used extensively in agriculture, horticulture, pest control, for industrial purposes, in vector control programs, plastic making, and for domestic purposes (Leong et al., 2018; Singh & Prasad, 2018; Ballantyne et al., 2017; Yadav et al., 2017; Eskenazi et al., 2014). Between 2011 and 2015, the worldwide use of OPs rose from 5 million to 6.8 million, as they are an alternative to POP OC pesticides. OPs degrade under natural conditions including sunlight, air and soil (Zhao et al., 2009). OPs are water soluble and diffuse into the environment by dissolution, abrasion and volatilization (Wang et al., 2014; Sidhu et al., 2019).

As with other pesticides, exposure routes for OPs include inhalation, absorption into the skin and ingestion (Jaga & Dharmani, 2003). OPs bind to cholinesterase (ChE) enzymes at the neuromuscular junction and deactivate or inhibit enzyme activity by irreversible phosphorylation. This in turn causes elevated levels of acetylcholine, which acts on the muscarinic receptors situated at cholinergic junctions in skeletal nerve-muscle junctions, at nicotinic receptors in the autonomic ganglia and receptors in the central nervous system (Chen et al., 2012). Red blood cell ChE activity and pseudo (or serum) ChE activity are inhibited by OPs.

Diet is a significant source of OP exposure (Fenske et al., 2002; MacIntosh et al., 2001). As with other pesticide classes, OPs are also regulated by the FSSAI in various food and dairy items. It is estimated that in India, 51% of food items are contaminated with pesticide residues and out of these 20% have residues which are above the maximum residue levels (Gupta, 2004). OPs have been found in various food items from across India. One study of vegetables from Hyderabad found chlorpyrifos in eggplants (24.02 µg/kg), cabbage (10.55 µg/kg), cauliflower (2.85 µg/kg), tomato (178.87 µg/kg) and okra (2.49 µg/kg). Similar trends were seen for acephate, fenitrothion and phosalone (Sinha et al., 2012).

3) Pyrethroids

Insecticides:	telfluthrin, permethrin-1, permethrin-2, L-cyhalothrin, cypermethrin-1, cypermethrin-2, cypermethrin-3, fenvelerate, deltamethrin, cyfluthrin
Herbicides:	pendimethalin

Synthetic pyrethroids entered the pesticide market after the reduction in OCs in the 1960s and subsequent introduction of OPs and carbamates. Although the compounds have a shorter half-life when compared to OCs, they do not accumulate in the environment. Synthetic pyrethroids are synthetic analogs and derivatives of pyrethrins and include a variety of over 1000 insecticides. Their production involves extensive chemical modifications which makes them highly toxic and less degradable in the environment. Pyrethroid pesticides act by disrupting sodium channels in the axons. These pesticides are sprayed over edible products to control pests and are also used as household insecticides and grain protectants.

They are grouped into types I and II, based on toxicological and physical properties. Type I pyrethroids are derivatives of pyrethrin that do not have a cyano group and result in tumors. Type II pyrethroids have a cyano group and cause chloreoathetosis and salivation (Thatheyus &

Selvam, 2013). Pyrethroids are generally composed of two, four or either isomers and their basic structure is composed of an acid, an alcohol moiety with an ester bond (Saillenfait et al., 2015). Upon uptake of pyrethroids into the human body, they are rapidly metabolized by hydrolysis of ester bond and by P-450 mediated oxidation. They have short half-lives and get excreted in urine as sulfate and glucuronide conjugates (Barr et al., 2010). In insects, pyrethroids work by modifying the kinetic voltage-sensitive sodium channels which mediate transient increases in sodium permeability of the nerve membrane that underlies the rising phase of the nerve action potential (Koureas et al., 2012).

Pyrethroid pesticides are less toxic as compared to OCs and OPs. Many SPs result in mild to severe irritation to the skin and eyes and some also cause facial skin sensitization. Exposure to pyrethroid pesticides occurs due to dietary intake and residential application of pyrethroids. Consumption of fresh and cooked fruits and vegetables has been linked to higher levels of exposure, as assessed by biological monitoring and analysis of pesticide residues in raw and processed food products (Fortes et al., 2013; Kimata et al., 2009; Lu et al., 2006; Melnyk et al., 2014; Reiderer et al., 2008).

Cypermethrin has been found in large concentrations in rain water from Hisar, India (1 µg/L), and these pesticides vaporize with water (Kumari et al., 2007). Pyrethroids have also been detected in bovine milk from Punjab at concentrations ranging between 0.5-0.9 ng/g (Bedi et al., 2015).

2.3.2 Regulatory limits

Maximum residue limits (MRL) are the maximum concentration of pesticide residues legally permitted in food or feed commodities (MacLachlan & Hamilton, 2010). These levels represent the maximum residue concentrations expected to be found in foodstuff or feed if a

pesticide is applied according to good agricultural practice or GAP (MacLachlan & Hamilton, 2010). MRLs comprise three components, namely, 1) residue definition, 2) sample definition (size and component to be analyzed/commodity, 3) numerical value. MRLs must be high enough so as to prevent chance exceedance, but not too high so as to prevent detection of misuse (MacLachlan & Hamilton, 2010).

The establishment of MRLs accounts for risk to consumers and such levels are not established for specific commodities if residues resulting from use of chemical product could represent an unacceptable risk to public health (MacLachlan & Hamilton, 2010). Food items are routinely monitored for MRL compliance and exceedance can result in economic and trade consequences. The primary purpose of MRLs is to establish a legally enforceable limit. Compliance with such limits should in turn, measure compliance with good agricultural practices (Renwick, 2002).

MRLs have been set by national and international regulatory bodies for different pesticides, classes of pesticides (by function) and food commodities (FAO/WHO, 2020). The Food Safety and Standards Authority of India (FSSAI) has set maximum residue levels (MRLs) for several OC pesticides in a variety of food items such as milk and milk products, vegetables and food grains (Food Safety and Standards Authority of India, Contaminants, Toxins and Residues Regulations, 2011). **Table 4**, below lists MRLs for a select set of pesticides across the three classes, in common food items set by JECFA/FAO and FSSAI.

Table 4: Maximum residue limits for pesticides set by JECFA/FAO and Food Safety & Standards Authority of India

Pesticides	Food Items	Regulatory Body (mg/kg)	
		JECFA/FAO (Codex Alimentarius)	FSSA India
DDT	Carrot	0.2	Banned from use in India
	Cereal grains	0.1	
	Eggs	0.1	
	Meat (mammals)	5	
	Milks	0.02	
	Poultry meat	0.3	
ΣDDT	Milk	0.05	
HCH	Milk	0.01	
Aldrin and Dieldrin	Cereal grains	0.02	
	Citrus fruits	0.05	
	Eggs	0.1	
	Leafy vegetables	0.05	
	Milks	0.006	
	Pulses	0.05	
Endrin	Fruiting vegetables	0.05	
	Poultry meat	0.1	
Endosulfan	Eggs	0.03	
	Meat(mammals)	0.2	

Endosulfan (cont'd)	Milks	0.01	
	Potato	0.05	
	Tomato	0.5	
Acephate	Eggs	0.01	-
	Meat	0.05	0.05
	Milks	0.02	0.02
	Rice	1	1
Chlorpyrifos	Eggs	0.01	-
	Milks	0.02	0.02
	Rice	0.5	0.5
	Sorghum	0.5	0.05
	Wheat	0.1	0.5
	Wheat flour		-
Malathion	Maize	0.05	0.05
	Sorghum	3	4
	Wheat	10	10
	Wheat flour	0.2	-
Cypermethrin	Eggs	0.01	-
	Leafy vegetables	0.7	-
	Milks	0.05	0.05

Cypermethrin (cont'd)	Rice	2	2
	Wheat	2	2
Cyfluthrin	Eggs	0.01	-
	Milks	0.01	-
	Potato	0.01	-
	Tomato	0.2	-

[a]CODEX Alimentarius Commission, 2020 (http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/en/)
[b]FSSAI, 2011 (https://tinyurl.com/ya33dzls)

2.3.3 Hazard identification & characterization and Exposure assessment

In relation to pesticides, the process of hazard identification involves in vivo and in vitro studies to define biological properties of chemicals that can lead to adverse effects if the doses are sufficiently high (Renwick, 2002). Hazard characterization is concerned with defining dose or concentration-response relationships in order to establish intake levels that would be without significant health effects in exposed humans (Renwick, 2002). The outputs of hazard characterization for pesticides are: 1) **Acceptable daily intake (ADI)**, which represents the amount of a chemical that can be consumed every day for a life-time, on the basis of all known facts, that no harm will result; 2) **Acute reference dose (ARfD)**, represents the amount of chemical that can be consumed at one meal or on one day, such that no harm will result, whereas **chronic reference dose (CRfD)** represents doses over a longer period of time ; 3) **Acceptable operator exposure level (AOEL)**, represents a level of daily exposure such that no adverse effects would result in operators who work with pesticides regularly, over a period of days, weeks or months (Renwick, 2002). **No-observed adverse effect level (NOAEL)** are derived based on the dose-response data for a potential adverse health effect of pesticides (Renwich, 2002; Bress, 2009).

Acceptable daily intake values have been set in addition to **oral and inhalation reference doses (RfDs in mg/kg/day)** for various insecticides, herbicides and fungicides. As noted above, ADI is defined as the level of daily intake for a toxic substance that does not produce an adverse health effect, whereas RfDs are regulatory parameters based on no-observed-adverse effect levels (NOAELs) (Watts, 2012). **Table 5** below, lists ADIs and references doses for several common pesticides.

Table 5: List of ADIs and reference doses for common pesticides

Pesticide(s)	ADI	PTDI	Acute RfD	Chronic RfD	Reference
ΣDDT p,p'-DDT + o,p'-DDT + p,p'-DDE + p,p'-TDE (DDD)	0.01 mg/kg bw				FAO/WHO, 2020
ΣDDT		10,000 ng/kg bw/day			FAO/WHO, 1995
HCH		5000 ng/kg bw/day			FAO/WHO
Endosulfan		6000 ng/kg bw/day			
Endrin		200 ng/kg bw/day			
Dieldrin		100 ng/kg bw/day			FAO/WHO, 2001
Cyfluthrin	40 µg/kg/day				FAOWHO
Deltamethrin	10 µg/kg/day				FAO/WHO

Fenvalerate	20 µg/kg/day				FAO/WHO
α-cypermethrin	20 µg/kg/day				FAO/WHO
Cypermethrin (adults, infants and children)	20 µg/kg/day	0-20,000 ng/kg bw/day	100 µg/kg/day	60 µg/kg/day	FAO/WHO USEPA, 2006
Permethrin	50 µg/kg/day		250 µg/kg/day		USEPA, 2009
Chlorpyrifos		0-10,000 ng/kg bw/day			FAO/WHO

[a]ADI = Acceptable daily intake; PTDI = provisional tolerable daily intake; Acute RfD = acute reference dose; Chronic RfD = chronic reference dose

2.3.4 Health outcomes

Pesticide exposure can occur via dermal, ingestion and inhalation routes of exposure and exposure has been linked to various diseases including cancers, asthma, Parkinson's disease, diabetes and leukemia (Kim et al., 2017).

OC toxicity is caused by stimulation of the central nervous system and cyclodienes such as the GABA antagonists, endosulphan and lindane inhibit calcium ion influx and Ca and Mg-ATPases, resulting in release of neurotransmitters (Matthews, 2015). With regards to longer term health outcomes, there is a growing body of evidence associating exposure to pesticides and diabetes. One systematic review and meta-analysis of 22 studies found associations between exposure to any type of pesticide (top vs. bottom tertile of exposure and diabetes, OR of 1.58 (95% CI: 1.32-1.90, $p < 0.001$) and diabetes (Evangelou et al., 2016). Results for type 2 diabetes, specifically, showed OR of 1.61 (95% CI: 1.37-1.88, $p < 0.001$). An increased risk of diabetes was seen for exposure to DDE, heptachlor, HCB, DDT and trans-nonachlor or chlordane, specifically, with mechanistic support for the link between OCPs and insulin resistance in the pathogenesis of diabetes (Evangelou et al., 2016). In a meta-analysis of 23 studies, Tang et al., (2014), found that exposure to OPs is associated with an increased incidence of type 2 diabetes, ORs for PCBs were (2.14, 95% CI: 1.53-2.99), and p,p'-DDE (1.33, 95% CI: 1.15-1.54), respectively. OC pesticides have been linked to breast cancer through potential estrogenic effect on mammary cells (Rivero et al., 2015).

Long-term low dose exposures are likely to result in immune-suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer (Gupta, 2004). OP toxicity in humans can occur due to acute or chronic exposure (King et al., 2015). OPs also pose potential risks for endocrine, metabolic, neurological, hepatorenal disorders and psychiatric

conditions in addition to neuritis (Kumar et al., 2016). Ventura et al., (2015) found that exposure to chlorpyrifos induces a redox imbalance that can alter antioxidant defense system in breast cancer cells.

Effects of chronic exposure to pyrethroids include cerebro-organic disorders, sensory-motor polyneuropathies in lower extremities, vegetative nervous disorders like paroxysmal tachycardia, increased heat sensitivity and reduced exercise tolerance related to circulatory dysfunction (Muller-Mohnssen, 1999). Studies have also found that pyrethroids may induce changes in immune mechanisms and neurodevelopment (Saillenfait et al., 2015).

A reduction in growth rate, liver enlargement and an increase in activity of some liver enzymes may be caused by synthetic pyrethroids. Immune system suppression, and damage to the nervous system can occur depending on the types of pyrethroids (Lu et al., 2006). Occupational exposure to fenvalerate can compromise the quality of semen and may also cause increased damage to sperm DNA (Bian et al., 2004). Permethrin has been reported as a potential or weak carcinogen by the USEPA (Thatheyus et al., 2013). More recently a large prospective study of permethrin-exposed pesticide applicators found no evidence for an increased risk of cancers, namely melanoma, leukemia, non-Hodgkin lymphoma or colorectal, lung and prostate cancers (Rusiecki et al., 2009). Urinary levels of pyrethroid metabolites may however be associated with elevated risk of childhood acute lymphocytic leukemia (Ding et al., 2012). Ntzani et al., (2013), in their meta-analysis of the literature found significant associations between pesticide exposure and childhood leukemia (OR: 1.69, 95% CI: 1.35, 2.11) and Parkinson's disease (OR: 1.58, 95% CI: 1.35-2.85, for general pesticide use; OR: 1.01, 95% CI: 0.78-1.30, for association with DDT exposure).

2.3.5 Association with pregnancy outcomes

Studies have examined the distribution of OC pesticides in the human body and concentration gradients of OCs between maternal and fetal compartments, and found that absorbed OCPs can cross the placental barrier and reach a balanced state between mother and fetus (Waliszewski et al., 2000). Studies have also shown positive associations between levels of OC pesticides in maternal sera and umbilical cord sera ($r = 0.421$, $p < 0.01$; $r = 0.288$, $p < 0.01$). DDT, β-HCH and HCB residues have been shown to bioconcentrate in maternal and cord sera, and correlation analyses have shown OC pesticides in maternal blood to transfer through the placenta and affect thyroid hormone levels in fetuses (Li et al., 2014). Pathak et al., (2008) have shown placental transfer rates of OCs to be between 60-70% from mothers to fetuses. Dewan et al., (2013) have also noted transplacental and trans-mammary transfer of OCPs.

Maternal Outcomes

Smarr et al., (2016), examined associations between maternal serum levels of POPs including OC pesticides. They found p,p'-DDE concentrations in serum at median levels of 0.57 ng/g (IQR: 0.41-0.78). These authors note that maternal preconception levels of OCPs were not significantly associated with *gestational diabetes mellitus*, although ORs were elevated in adjusted models for oxychlordane (OR = 1.26, 95% CI: 0.76-2.08) and p,p'-DDE (OR = 1.20; 95% CI: 0.72-2.02). γ-HCH was significantly associated with *gestational hypertension*, in this study after fully adjusting for low-dose additivity of other OCPs (OR = 1.41, 95% CI: 1.01-1.96) (Smarr et al., 2016). Warembourg et al., (2019) examined whether exposure to environmental chemicals increases susceptibility to higher blood pressure among pregnant women, as part of the Human Early-Life Exposome (HELIX) project. These authors found that exposure to OPs

was not associated with an increase/decrease in blood pressure during pregnancy (Warembourg et al., 2019).

Tyagi et al., (2015), examined the xenoestrogenic effects of OC pesticides and their associations with preterm birth through disturbances to normal estrogen-progesterone ratios, in a small sample of women from Northern India. These authors noted that significantly higher levels of α-hexachlorocyclohexane (α-HCH), β-HCH, DDD and DDE were found in the blood of mothers who experienced preterm birth when compared to controls. This was in addition to significantly higher levels of DDE and DDT in placental tissue of preterm birth cases, as compared to controls (term deliveries). Statistically significant negative correlations were observed between maternal blood levels of α-HCH and birth weight ($r = -0.299$) and period of gestation ($r = -0.234$). γ-HCH and dieldrin were negatively correlated with placental weight ($r = -0.401$ and -0.256) and DDE and β-HCH negatively correlated with period of gestation ($r = -0.251, -0.299$). OCPs, due to their chronic bioaccumulation and poor elimination can pose a risk to fetuses and lead to harmful health effects (Tyagi et al., 2015).

POPs are transferred by passive diffusion between maternal and infant compartments, depending on lipid percentage, and ratios between compartments are expected to be close to one. Muller et al., (2019) have found strong correlations between levels of p,p'-DDE in maternal blood and cord blood, this also indicates placental transfer of these compounds. These findings suggest that p,p'-DDE may also build up and equilibrate between fetal compartments. Lower fetal detoxification processes and lower excretion capacity, as compared to the mother, may contribute to higher concentrations of p,p'-DDE in cord blood (Waliszweski et al., 2001; Muller et al., 2019).

Exposure to OP pesticides during pregnancy have been associated with maternal endocrine disruptions and disturbed hepatic function. In a prospective study among n = 97 pregnant women, from Brazil, blood biomarkers of OP exposure, namely cholinesterases and β-glucuronidase, cortisol and progesterone levels, as well as glycemia were detected. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), markers of liver injury and albumin as a marker of liver function were also assayed. These authors found cortisol levels to be higher in the maternal compartment, likely due to hormone metabolism pathway changes driven by exposure to OPs, which may in turn lead to impaired newborn health later in life. Subclinical hepatoxicity was also indicated among women during the second trimester of pregnancy by increases in alanine transaminase (ALT) values and (aspartate aminotransferase) AST/ALT ratio (Cecchi et al., 2012).

Child Outcomes

Various studies have examined associations between pesticide exposure and pregnancy and birth outcomes. A study conducted in China examined *in utero* exposure to 18 organochlorine pesticides (OCPs) including DDT, HCH, HCB, heptachlors, chlordanes, endosulfan and mirex, in 81 mother-infant dyads. These authors found that a 1 ng/g increase in cord serum levels of p,p'-DDE, total DDT and β-HCH was associated with 0.1g, 0.1g and 0.92 g decrease in infant birth weight, respectively (Guo et al., 2014). Similarly, Siddiqui et al., (2003), found statistically significant associations ($p < 0.05$) between maternal blood levels of OCs, α-HCH (OR = 1.22, 95% CI: 1.02-1.46), γ-HCH (OR = 1.38, 95% CI: 1.05-1.80), δ-HCH (OR = 1.31, 95% CI: 1.00-1.75), and total HCH (OR = 1.07, 95% CI: 1.00-1.14) and intra-uterine growth restriction, after adjusting for potential confounders (Siddiqui et al., 2003). These authors found a significant negative correlation between body weight of newborn infants and p,p'-DDE

in maternal blood ($r = -0.25$, $p < 0.05$) and δ-HCH and p,p'-DDE in cord blood ($r = -0.27$, and -0.26, $p < 0.05$), after adjusting for gestational age among Indian women (Siddiqui et al., 2003). Pathak et al., (2009) found statistically significant associations between preterm birth and β-HCH in maternal and cord blood, but also found α-HCH, β-HCH, γ-HCH, total HCH, p,p'-DDE and p,p'-DDT to be higher among women who experienced preterm labor compared to full term births (Pathak et al., 2009).

In an American study, Longnecker et al., (2001), found maternal DDE concentrations at median value of 25 µg/L, with adjusted odds ratios for preterm birth increasing steadily with an increase in concentrations of maternal serum DDE (OR = 1, 1.5, 1.6, 2.5, 3.1, $p < 0.0001$). Adjusted ORs for small-for-gestational age also increased (OR = 1, 1.9, 1.7, 1.6, 2.6, $p = 0.04$).

Negative associations were also noted between length at birth and t-HCH levels in cord blood, breastmilk, β-HCH in cord blood and breastmilk. OCPs were also found to be negatively correlated with head circumference, ponderal index and chest circumference in neonates (Dewan et al., 2013). Dewan et al., (2013) have noted significant negative correlations between birthweight and t-HCH, β-HCH, γ-HCH and DDT levels in maternal blood, cord blood, placenta and breastmilk ($p = 0.005$).

Children and infants are particularly vulnerable to OP exposure, and the extent of OP toxicity in children is determined by their general health, nutritional status, metabolic rate, immune status and the dose of exposure (Lewis et al., 2001). Placental transfer of OPs has been documented in animal models and in-utero exposure can affect newborn children and infants (Astroff et al., 1998). Maternal exposure to OPs can affect acetylcholinesterase in the fetal brain and in turn impact fetal neural function (Banerjee et al., 1991).

Pooled results from four cohort studies have examined associations between prenatal OP exposure, birth weight (n = 1,169), length (n = 1,152) and head circumference (n = 1,143). Concentrations of OP pesticides were measured in maternal urine samples. These authors found no significant associations between ΣDEP (diethyl phosphate), ΣDMP (dimethyl phosphate), or ΣDAP (dialkyl phosphates) and birth weight, length or head circumference. Among non-Hispanic black women, associations were observed between increased urinary ΣDAP and ΣDMP and decreased birth length (β = -0.4 cm; 95% CI: -0.9-0.0 and β = -0.4 cm; 95% CI: -0.8-0.0, for each 10-fold increase in metabolite concentration) (Harley et al., 2016). More recently, Yang et al., (2020), have found prenatal exposure to β-HCH to be associated with decreased birth weight via disruption of thyroid hormone and glyceraldehyde metabolism (Yang et al., 2020).

A large case-control study conducted in North Carolina between 2003-2005 included women with infants of gestational ages 20-44 weeks (n = 304, 906), and assessed pesticide exposure using a chemical exposure metric based on planting/harvesting dates for crops. These authors used logistic regression analyses and found higher odds of congenital heart defects, certain structural defects associated with gastrointestinal, genitourinary and musculoskeletal systems among highest exposed versus unexposed. Additionally, OR of 1.98 (95% CI: 0.69-5.66) was found for tracheal esophageal fistula/esophageal atresia and OR of 1.70 (95% CI: 1.34-2.14) was seen for atrial septal defects (Rappazzo et al., 2016). Muller et al., (2017) have found that p,p'-DDE in breastmilk samples from mothers in Tanzania to be negatively associated with head circumference among female children.

2.3.6 Breastmilk and infant foods

Pesticides can enter the child's food supply via breastmilk (Mead, 2008; Mapesa et al., 2015). OC, OP and pyrethroid pesticides have been extensively documented in breastmilk samples from across the globe and several factors have been associated with levels of various pesticides in breastmilk, including maternal age, parity, BMI, maternal % fat mass (Pirsaheb et al., 2015; Salama, 2017).

A study in Tanzania found DDTs and dieldrin in breastmilk samples at ranges between 24-2400 ng/g lipid weight and <LOD-157 ng/g lw, respectively (Muller et al., 2017). Dieldrin was found at levels up to 937 ng/g lw in this study. Du et al., (2017) tested for OCPs, OPPs, carbamates and pyrethroids in breastmilk samples from Australia and found p,p'-DDE in 87% of samples in the tested cohort at mean concentrations (± SD) of 1.9 ± 1.9 ng/mL (range: 0.2-10.1 ng/mL) and 62.8 ± 54.5 ng/g fat (range: 6.3-209.2 ng/g fat).

Pyrethroid pesticides including cypermethrin, ƛ-cyhalothrin, permethrin, esfenvalerate/fenvalerate have been detected at concentrations ranging between 1.45-24.2 ng/g lipid weight, in samples from Brazil, Colombia and Spain (Corcellas et al., 2012). Bouwman et al., (2006) found mean Σpyrethroid concentrations in breastmilk samples at 31.5 µg/L from malaria endemic areas in KwaZulu-Natal, South Africa. In Mexico, Limon-Miro et al., (2017), found statistically significant differences in concentrations of p,p'-DDT in agricultural vs. urban areas (30 ± 200 vs. 46 ± 900, p = 0.008), in addition to cypermethrin (3 ± 1.3 vs. 6.3 ± 24, p = 0.001) and cyhalothrin (8.6 ± 3.1 vs. 1.86 ± 780, p = 0.08).

Studies conducted in India have detected OCs in breastmilk samples from various parts of India. Devanathan et al., (2009), found DDTs to be the predominant contaminants in human milk samples from mothers in 4 major urban cities in India, namely New Delhi, Mumbai,

Kolkata and Chennai. DDTs were detected at concentrations ranging between 47-1200 ng/g lipid weight, followed by HCHs (6.3-1900 ng/g lw), CHLs (0.048-21 ng/g lw) and HCB (0.4-19 ng/g lw). These authors note based on their findings and those of others, that DDTs are the major contaminants in northern, western and eastern parts of India. High levels of DDTs are likely seen in these studies due to their use in vector control programs for malaria. DDT was banned for agricultural use in 1989, but is used for public health purposes (restricted use up to 10,000 tons/annum), due to cost effectiveness, multi-spectrum applicability and persistence (UNIDO, 2006). Elevated levels of DDTs have been found in human milk samples from Mexico (Waliszewski et al., 2001), Thailand (Stuetz et al., 2001), China (Wong et al., 2005) and Vietnam (Minh et al., 2004). Levels of DDTs seen in India are in concordance with levels observed in developing countries such as Indonesia (Sudaryanto et al., 2006), Malaysia (Sudaryanto et al., 2005) and Cambodia (Kunisue et al., 2004). Levels are one order of magnitude higher than those seen in industrialized nations such as Japan (Kunisue et al., 2006), UK (Harris et al., 1999), Sweden (Noren and Meironyte, 2000), Germany (Schoula et al., 1996) and Canada (Newsome & Ryan, 1999).

A study conducted in Madhya Pradesh, India has shown HCH isomers (0.036 and 00.004 mg/L) and β-HCH isomers (0.064 mg/L) in human breastmilk, in addition to endosulfans (0.61 g average daily intake) (Sanghi et al., 2003). Effects of chronic exposure to DDTs, HCH, endosulfans and chlorpyrifos include decreased fertility, increase in birth defects and neonatal deaths (Sanghi et al., 2003).

Bedi et al., (2013) found HCHs at concentrations of 199.6 ng/g lipid weight and DDTs at 1914.2 ng/g lipid weight from breastmilk samples in Punjab. HCH isomers including γ-HCH and β-HCH were seen at 101.7 ng/g and 97.9 ng/g lipid wt, respectively. Individual residues of *p,p'*-

DDE were seen at mean concentrations of 1574.0 ng/g lw, *p,p'-DDD* at 239.8 ng/g lw and *p,p'-DDT* at 100.3 ng/g lw, respectively (Bedi et al., 2013). These authors also detected endrin, β-endosulfan and endosulfan sulfate at mean concentrations of 107.64, 90.69 and 14.00 ng/g lipid wt. Pyrethroid pesticide, Cypermethrin was detected at 84.15 ng/g lipid weight. Other non-persistent pesticides including endosulfan, chlorpyrifos and cypermethrin were also detected in Indian human breast milk samples. Recent studies in India have shown concentrations of POPs including DDT (490 ng/g) and HCH (46.6 ng/g) in human breastmilk from Punjab, India (Bawa et al., 2018).

Few studies have quantified levels of pesticides in infant formula samples from India. Mishra et al., (2002) found various pesticide residues in commercial infant formula products including α-HCH at mean [1.85 µg/kg], χ-HCH at mean [0.88 µg/kg], β-HCH at mean [86.6 µg/kg], δ-HCH at mean [0.71 µg/kg], α-endosulfan at mean [2.68 µg/kg], DDE at mean [0.86 µg/kg], Dieldrin at mean [0.33 µg/kg], DDD at mean [1.21 µg/kg], β-endosulfan at mean [0.38 µg/kg], DDT at mean [0.74 µg/kg], malathion at mean [10.51 µg/kg]. Acceptable daily intake levels for DDT (Gunderson, 1995) are calibrated at 20 µg/kg body weight for adults for DDT. This study showed that mean concentrations for DDT would exceed 50 µg/kg per pesticide for a child with mean body weight of 5 kg and mean intake of 0.5 kg of infant solid food (Mishra et al., 2002). In addition to infant formula, Sharma et al., (2007) found mean values of ΣHCH, ΣDDT, Σendosulfan and aldrin at 0.0292, 0.0367, 0.0022 and 0.0036 µg/mL, respectively, in bovine milk collected from 14 districts of Haryana, India. Several samples exceeded FAO/WHO set MRLs for ΣHCH (0.1 mg/kg), ΣDDT (0.05 mg/kg) in this study.

2.3.7 Associations with child growth outcomes

Children are particularly vulnerable to the effects of pesticide exposure because of the incomplete nature of the detoxification process, age related speed in neurodevelopment and increased exposure relative to their pound of body weight (Eskenazi et al., 1999; Fiedler et al., 2015). Few studies have examined the associations between pesticide exposure and infant growth outcomes, although the evidence of the effects of pesticides on neurodevelopment is substantial and mounting (Liu et al., 2012; Rauh et al., 2006; Bouchard et al., 2010).

Studies from Spain have shown serum IGF-1 levels in boys 6-15 years of age who are exposed to OCPs to be lower than among unexposed boys (Zumbado et al., 2010), and low serum IGF levels have been shown to be associated with growth disorders (Idohou-Dossou et al., 2003). As pesticides are endocrine disrupting chemicals, human exposure can interfere with hormones such as thyroid hormones, insulin and IGF-1, all of which are involved in the growth process (Wallace et al., 2003; Idohou-Dossou et al., 2003). Deficiencies in thyroid hormone can also cause metabolic disorders which result in growth and developmental disorders (Setian, 2007). Exposure to pesticides can cause thyroid dysfunction through mechanisms that disrupt TSH receptors on the thyroid gland, particularly for pesticides that have structures similar to thyroid hormone. This leads to reductions in D1 (deiodinase type 1) enzyme activity, and D3 enzyme stimulation (Boas, Feldt-Rasmussen et al., 2006). Additionally, OCs also impact IGF-1 activity, and Kartin et al., (2019) found that children with low levels of IGF-1 had 8.35 times higher risk for stunting compared to normal IGF-1 levels. IGF-1 mediates protein anabolic processes and increased growth hormone activity (Skottner, 2012; Larson, 2001). These authors also noted that after adjusting for confounding, low IGF-1 levels (aOR: 8.35, 95% CI: 3.65-

19.14) and a history of pesticide exposure (aOR: 3.90, 95% CI: 1.15-13.26) were independently associated with stunting in children 8-12 years of age (Kartin et al., 2019).

As noted by Reinhardt and Fanzo (2014), limited evidence exists showing biological impact of pesticides on growth in humans. *In vivo* studies using rat models have shown that OP metabolites in the gut lead to elevated levels of oxidative stress and inflammatory markers including tumor necrosis factor (TNF-α), interleukin-6β and nuclear factor (NF)-κB (Ghasemi-Niri, Maqbool et al., 2016). Furthermore, studies in mice have shown chronic exposure to OP pesticides such as chlorpyrifos leads to increased permeability of the epithelial barrier of the small intestine, particularly during weaning, when the digestive tract is immature (Condette, Khorsi-Caudet et al., 2014). OPs and carbamates work by inhibiting cholinesterase, an enzyme responsible for the breakdown of acetylcholine (Chen et al., 2014). It is unclear whether this may play a role in the development of a "leaky gut" however, paralysis of the gut lining is a common symptom in OP poisoning (Chen et al., 2014). Pesticide exposure may be a contributing factor to the development of a leaky gut in the mother and through transmission in breastmilk to the child, as well. It is unclear how pesticides impact the inflammatory profile of breastmilk.

Mapesa et al., (2016), have postulated that dietary exposure from breastmilk, food and water to multiple classes of pesticides can contribute to environmental enteric dysfunction. Given that xenobiotic pesticides affect physiology, metabolism and gene expression of the human gut microbiome, additional research is needed to understand the interactions between xenobiotics and the gut microbiome. Exposure to pesticides also leads to oxidative stress, which in turn increases energy expenditure and activation of the immune system, diverting essential energy resources away from maintenance, reproduction, growth and thermoregulation (Degen et al., 2006; Mostafalou et al., 2013).

In a study of Australian breastmilk, Du et al., (2017), found no significant associations between p,p'-DDE concentrations in human milk samples and infant weight, body length, head circumference, and percentage fat mass during breastfeeding. Additionally, no significant associations were observed between detected p,p'-DDE and infant growth outcomes in this cohort, after using more specific measures including ultrasound and bioimpedance spectroscopy (BIS). Other studies that have examined relationships between HM and infant birth outcomes such as birth weight, length and chest circumference with prenatal POP exposure, however these studies have failed to yield consistent results (Eskenazi et al., 2004; Whyatt et al., 2004; Wolff et al., 2007; Rosas & Eskenazi, 2008).

Exposure to OPs such as dimethylphosphate (DM), diethylphosphate (DE) and total dialkylphosphate (DAP) metabolites in urine among pregnant women in China and their children at 2 years of age were examined and correlated with neurodevelopment at 2-years of age (Liu et al., 2016). These authors found that pre and postnatal OP exposure was significantly associated with an increased risk of development delays, with ORs for prenatal DE exposure and adaptive neurodevelopment of 9.75 (95% CI: 1.28-73.98, p = 0.028). ORs for social area neurodevelopment and postnatal exposure to DEs and DAPs were 9.56 (95% CI: 1.59-57.57, p = 0.014) and 12.00 (95% CI: 1.23-117.37, p = 0.033), respectively. These associations were observed primarily among boys (Liu et al., 2016).

Overall studies of pesticides in human milk and other samples are heterogenous in their results with regards to infant growth. Longitudinal studies that assess biomarkers from various samples at many time points during pregnancy, lactation and beyond are needed. These studies should account for urban and rural communities and for confounders including maternal anthropometry, socioeconomic status and all potential exposure sources. Additional studies

should also examine the intergenerational risks of exposure to pesticides and their links to the epigenome, metabolome and exposome. An examination of interactions between various environmental toxicants such as mycotoxins and pesticides, dietary exposures and poor sanitation are prudent, as they all contribute to transient and chronic environmental enteric dysfunction, resulting in infant growth faltering and stunting.

2.4 Methods for Assessment of Mycotoxins & Pesticides

Biomarkers are biological markers that allow for the measurement and evaluation of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Atkinson et al., 2001). The use of biomarkers for measurement of exposure to xenobiotics is commonplace, and utilizes estimations of metabolites in biological fluids i.e. biomarkers of exposure. Biological fluids used to analyze biomarkers include urine, blood, breastmilk, hair and feces (Sewram et al., 2001, Shephard et al., 2007; Tozzi et al., 2016). Exposure biomarkers are also classified as biological measures which are correlated to the quantity of a xenobiotic ingested, resulting in improved exposure classification, as opposed to a bio-measure which only accurately quantifies the amount of a toxin and/or metabolite, but is not correlated to dietary intake (Turner et al., 2012).

Several different methods have been developed to detect and quantify biomarkers in human milk including xenobiotic compounds such as mycotoxins and pesticides. The most prominent and effective of these include ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and gas chromatography tandem mass spectrometry (GC-MS/MS) (Ballard & Morrow, 2013; Wu et al., 2018). It is important to note that differences in levels of reported mycotoxins and pesticides in breastmilk are highly dependent on the analytical methods used and their corresponding **limits of detection (LOD) and quantification (LOQ)**.

Limit of detection of an analytical method is defined as the lowest quantity or concentration of an analyte that can be reliably detected, with a signal-to-noise ratio ≥ 3 (& %RSD of 33%), whereas the limit of quantification is the lowest concentration that can be measured with an intra-assay precision cost variance and relative bias < 20%, and signal-to-noise ratio > 10 (& %RSD of 10%) (Armbruster et al., 2008).

This section will discuss biomarkers of exposure, different assays, extraction protocols, detection methods and end with a comparison of LODs and LOQs for these methods and implications for quantification of trace substances such as mycotoxins and pesticides in milk matrices.

Mycotoxins

2.4.1a Biomarkers and matrices

Several biomarkers have been used to study mycotoxin exposure in humans. Metabolism of aflatoxins gives rise to a variety of metabolites, which include **AFM_1**, which is frequently detected in the milk of lactating animals and humans, followed by maternal dietary consumption of AFB_1 (Turner et al., 2012; IARC, 1993; Eaton et al., 1994; Zarba et al., 1992). Parent toxins, AFB_1 and AFG_1 are also detected in breastmilk (IARC, 1993; Polychronaki et al., 2007; Polychronaki et al., 2006). **Urinary** biomarkers of aflatoxin exposure include **AFB_1-N7-guanine**, which results from metabolism of AFB_1 by cytochrome P450 and generation of two reactive epoxide species, an exo-epoxide and endo-epoxide (Abia et al., 2013). Covalent bonding of the N7 moiety of guanine and the exo-epoxide result in release of **AFB_1-N7-guanine**, which has been found to be strongly correlated with aflatoxin intake ($r = 0.80$, $p < 0.0001$; and $r = 0.82$, $p < 0.0001$), in addition to **AFM_1** in urine ($r = 0.82$, $p < 0.0001$) (Groopman et al., 1992; Zhu et al., 1987). In serum, **aflatoxin-albumin** is correlated to aflatoxin intake in diet ($r = 0.69$, p

<0.0001), and is another widely used exposure biomarker (Gan et al., 1988; Wild et al., 1992), **AFB_1-lysine adducts** is the major protease product in sera.

OTA in milk, blood serum, urine and in human kidneys are classified as valid biomarkers of exposure to OTA (Malir et al., 2016). Fumonisins are not extensively metabolized and are hydrophilic, therefore measurement of the parent compound concentrations in bio-fluids is widely used (Turner et al., 2012). **Urinary FB_1** has been measured in Mexican women with frequent tortilla consumption, where 74.6% of women had detectable urinary FB_1 (> 20 pg/mL) (Gong et al., 2008). Other studies conducted in South Africa have also found associations between estimated FB_1 intake/kg BW per day and urinary FB_1 adjusted for creatinine (van der Westhuizen et al., 2011). Urinary FB_1 is therefore regarded as the exposure biomarker for fumonisins. **Deoxynivalenol** is measured in urine as deoxynivalenol and deoxynivalenol-glucuronide, DON-3-glucuronide and DON-15-glucuronide have also been validated as suitable biomarkers of DON in addition to DON-3-glucoside in urine (Turner et al., 2012; Vidal et al., 2018). The objective of our work was to quantify aflatoxins and ochratoxins in human breastmilk and food samples, the following sections will therefore focus on assays and methods to detect assorted mycotoxins including AFs, OTs, FBs and DON in milk and assorted food commodities.

As regulatory limits have been put in place for AFs in milk and milk products, methods must be able to detect these low levels of contamination to ensure compliance with standards. As breastmilk is not regulated, no limits exist for this matrix, however comparisons can be made with maximum tolerance levels set for commercial milk and infant formula products, which have been instituted by several international regulatory agencies, as described earlier (Zhang et al., 2018).

2.4.2a Sample preparation and analytical methods

Sample preparation

Extraction procedures used to remove mycotoxins from milk matrix depend on the structure of the toxins. Polar molecules require water and organic solvents, as is the case with FBs. AFs which are hydrophobic, require organic solvents (Turner et al., 2009). The main component of an assay is the clean-up protocol which can take upwards of 2/3 of processing time and is considered the most important step. Matrix effects caused by interfering components of the matrix, particularly proteins and other compounds may mask the molecule of interest (Turner et al., 2008). Human milk in particular is composed of a mixture of essential nutrients such as proteins, carbohydrates, fats, vitamins and minerals, which may create interferences and lead to signal suppression (Rubert et al., 2014). Sample preparation methods include liquid-liquid extraction (LLE), immuno-affinity cartridges (IAC) and solid-phase extraction (SPE) clean-up protocols (Warth et al., 2016). Some researchers have also utilized QuEChERS method for analysis of mycotoxins in food matrices (Rubert et al., 2012). Liquid extraction or partitioning and protein precipitation in milk are the simplest protocols for mycotoxin analysis in milk samples.

Briefly, LLE, exploits the different solubilities of toxins in aqueous phase and in immiscible organic phase, to extract compounds into one solvent, leaving the rest of the matrix in the other. Hexane and cyclohexane are often used to remove non-polar contaminants such as lipids and cholesterol. This procedure, although time consuming and dependent on the matrix works well for small-scale preparations (Turner et al., 2009).

Solid-phase extraction (SPE) is designed on the principles of chromatographic techniques and uses small disposable cartridges packed with silica gel or bonded phases which are in the

stationary phase. The sample is loaded in one solvent, under reduced pressure, rinsed so contaminants are removed and eluted into another solvent (Turner et al., 2009). The SPE cartridge can bind small molecules. C-18 columns are typically used for reverse phase SPE, which is useful for non-polar and hydrophobic molecules, in biological matrices such as breastmilk, serum, plasma and urine. SPE protocols are time consuming, require additional equipment and solvents for extraction.

The QuEChERS extraction method also described as quick, easy, cheap, effective, rugged and safe, has also been used for detection of mycotoxins and pesticides in breastmilk (Rubert et al., 2014). This process involves solvent clean-up that uses extraction in acetonitrile, followed by salting out, a quick SPE step with simple materials ($MgSO_4$, primary secondary amine (PSA)), C18 and alumina), to remove matrix and then direct injection (Turner et al., 2015). Following sample extraction, various enzyme-linked and chromatography-based methods can be used for identification, quantitation and determination of mycotoxins.

Separation and detection methods

A systematic review of the literature on mycotoxin detection in breastmilk noted the use of various technologies, of which 70% of studies utilized high performance liquid chromatography “HPLC” to quantify mycotoxins in breastmilk, whereas 25% have used enzyme linked immunosorbent assays (ELISA) (Cherkani-Hassani et al., 2016).

Other methods include thin-layer chromatography (TLC), high-performance liquid chromatography-fluorescence detection (HPLC-FD), liquid chromatography tandem mass spectrometry (LC-MS/MS), gas chromatography (GC-MS) and enzyme-linked immunosorbent assays (ELISA) (Mwanza et al., 2015). TLC based methods are laborious and require large quantities of solvent and matrix (Dutta & Das, 2011). ELISA based high throughout approaches

can generally detect single analytes, however require validation for matrices such as breastmilk. The main principle behind this method is interaction of antigens and antibodies. This method offers high sensitivity and specificity (selectivity) to certain types of pesticides (Samsidar et al., 2018). ELISAs are available as commercial kits, validated and routinely used for analysis of food samples, as will be presented in chapter 4 of this dissertation.

The use of ultra-high-performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry has several benefits which include short run times, improved sensitivity and specificity, particularly for detection of multiple analytes of similar molecular structures and improved peak resolution (Aguilera-Luiz et al., 2011). Additional benefits include the quantitation and confirmation of the presence of multiple mycotoxins at trace concentrations (femtograms/L and above) (Zhang et al., 2018). An important consideration with regards to biomonitoring is the volume of biological matrix available for analysis. Lower volumes are preferred, particularly for biomarker analyses which may involve invasive extraction of bodily fluids, or non-invasive matrices such as breastmilk. High resolution methods also benefit from the need for smaller volume of matrix. This in turn, reduces the amount of time and effort required for extraction and cleanup steps.

As described earlier, matrix effects are an important consideration when dealing with biological matrices, and can result in signal suppression and enhancement caused by coeluting matrix components (Sfroza et al., 2006; Trufelli et al., 2011). These effects can vary by sample preparation (extraction and cleanup protocols), LC separation (mobile phase, flow rate and gradient), ionization source, and matrix-analyte combinations. The application of stable isotope dilution (SIDA), or use of deuterated or isotopically labelled internal standards to minimize the impact of matrix effects on quantitation of multiple analytes using mass spectrometry is also

gaining prominence (Asam & Rychlik, 2007). Few studies have applied SIDA LC-MS/MS for determination of mycotoxins in milk (Li et al., 2011; Zhang et al., 2013). It is important to note that these ISTDs are costly and hard to procure, despite their desirability for use in assays.

For the purposes of this dissertation, I will briefly discuss below, recent methods that have used ultra-high-performance liquid chromatography for mycotoxin analysis in breastmilk and compare and contrast method specifications.

2.4.3a Comparisons of LODs, LOQs and method performance

Most methods to date have focused on detection and quantification of a single analyte or single class of mycotoxins in human breastmilk (Warth et al., 2016), although the emerging literature shows application of methods to detect multiple classes of mycotoxins (Andrade et al., 2013; Rubert et al., 2014; Braun et al., 2018; Tonon et al., 2018; Zhang et al., 2018; Braun et al., 2020). As discussed earlier, AFM_1 and OTA are the primary mycotoxins found in breastmilk, however recent papers have quantified several classes of mycotoxins using a single method (Warth et al., 2016). **Table 6** below provides a comparison of recent UHPLC-MS/MS based methods for quantification of mycotoxins in human breastmilk samples.

The method we describe in chapter 3, although modelled off those in the existing literature, has LODs and LOQs that are on par if not lower than those in the extant literature, with need for lower volume of milk and easy sample processing. As discussed by Warth et al., (2016), there is a move towards development and use of multianalyte methods based on the use of advanced LC-MS/MS and LC-HRMS instrumentation. Although not extensively used at present for breastmilk analysis, time of flight (TOF) and orbitrap high resolution mass spectrometry instruments which allow full scan mode, have higher sensitivity, and superb mass

accuracy are predicted to be the gold standard in the future of breastmilk biomonitoring for mycotoxins (Warth et al., 2016).

Table 6: Comparison of recent methods for detection of multiple mycotoxins in animal and human milk samples

Mycotoxin	Samples analyzed (N) & Positive samples (n, %)	Concentrations		Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs	Extraction protocol & analytical method	Reference
		Concentration range	N				
AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, OTA, T-2 toxin	N: 15	ND	ND	AFM1: 84.5 (9.3)	LOD and LOQ for AFM1: 0.01, 0.03 µg/kg	• 10 mL milk • SPE • UHPLC-QqQ-MS/MS	• Aguilera-Luiz et al., (2011) • Method for pesticides and mycotoxins • Animal milk • Spain
AFB1, AFB2, AFG1, AFG2, AFM1, OTA	N: 224 N (%): 7	AFB2 @ LOQ (0.005 ng/mL)	2	Calibration curve: AFM1, AFG1, AFB1, OTA: 0.2-15 ng/mL AFG2, AFB2: 0.06-5 ng/mL Rec: 57-82.3% RSD: 3.8-17.3% (LOQ)	LOQ: AFM1, AFG2, OTA: 0.01 ng/mL AFG1: 0.03 ng/mL AFB1: 0.02 ng/mL AFB2: 0.005 ng/mL	• 2 mL milk • LLE-LTP (low temperature purification) • HPLC-FLD • + samples confirmed in LC-MS/MS (400 QTRAP triple quad mass spec)	• Andrade et al., (2013) • Brazil • Mature human milk
NIV, DON, 3-ADON, FUSX, **NEO**, DAS, T-2, **HT-2**, **ZEA, α-ZOL**, **β-ZOL**, FB1, FB2, FB3, **ENA, ENA$_1$, ENB, ENB1**, BEA, AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$, AFM$_1$, STER, OTA, OTα	N: 35 n (%): 60	ZEA: 2.1-14.3 ng/mL αZOL: 16.7 ng/mL βZOL: 39.8 ng/mL HT-2: 12.2-62.5 ng/mL NEO: 11.1-36.9 ng/mL ENA: 20.1-25.2 ng/mL ENA1: 42.1-51.1 ng/mL ENB: 99.8-110.3 ng/mL ENB1: 90.7-101.1 ng/mL NIV: 53.1-69.7 ng/mL	13 1 1 10 7 2 2 2 2 3	Calibration curve: LCL – 100x LCL Rec: 64-93% RSD: < 20%	Lower calibration limit: 1-50 ng/mL	• 10 mL milk • Simplified QuEChERs procedure • UHPLC-HRMS (orbitrap mass spec)	• Rubert et al., (2014) • Spain • Mature human milk

Table 6 (cont'd): Comparison of recent methods for detection of multiple mycotoxins in animal and human milk samples

Mycotoxin	Samples analyzed (N) & Positive samples (n, %)	Concentrations		Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs	Extraction protocol & analytical method	Reference
		Concentration range	N				
Aflatoxins, Beauvericin, Citrinin, Deoxynivalenol, Enniatins, HT-toxin, nivalenol, Ochratoxins, Sterigmatocystin, T-2 toxin, zearalenones	N = 75	AFM1: < LOQ OTA: < LOQ	1 11	Calibration curves: AFM1: 0.05-30 ng/mL OTA: 0.1-60 ng/mL AFM1: RE±RSD: 91±13 OTA: RE±RSD: 96±5	LOD, LOQ AFM1: 0.043, 0.087 ng/mL OTA: 0.048, 0.096 ng/mL	• 2 mL milk • QuEChERS - salting out $MgSO_4$ and NaCl • ACN layer frozen and filtered (PTFE) • LC-MS/MS (TSQ triple quad mass spec)	• Braun et al., (2018) • Human mature milk • Nigeria
AFM1, OTA, DON	N: 86	All < LOD	-	RE±RSD: 121±11 RE±RSD: 133±14 RE±RSD: 110±11 (v. low)	LOD, LOQ AFM1: 0.00625, 0.0125 ng/mL OTA: 0.125, 0.25 ng/mL DON: 2.5, 5 ng/mL	• 10 mL milk • C18-SPE • LC-MS/MS (QTRAP 5500)	• Tonon et al., (2018) • Human milk • Brazil
AFB1, AFB2, AFG1, AFG2, AFM1	N: 3 CRM milk powders	AFM1: 0.106 ng/g (283 ref) AFM1: 0.61±0.07; 0.45±0.03; 0.44±0.06 ng/g (284 ref)	-	Calibration curves: 0.01-50 ng/mL Rec: 80-120% RSD: < 20%	LOD, LOQ AFB1: 0.10, 0.47 ng/g AFB2: 0.24, 0.31 ng/g AFG1: 0.24, 0.33 ng/g AFG2: 0.19, 0.40 ng/g AFM1: 0.12, 0.38 ng/g	• 0.5-1 gm • Protein precipitation • ISTDs for SIDA ^{13}C • LC-MS/MS (SCIEX QTrap)	• Zhang et al., (2018) (AOAC) • Whole milk, milk-based infant formula, animal feed
34 mycotoxins including Aflatoxins, Alternariol, alternariol monomethyl ether, Beauvericin, Citrinin, Deoxynivalenol, Enniatins, Fumonisins, HT-2 toxin, Ochratoxins, Sterigmatocystin, T-2 toxin, tentoxin, zearalenones	N: 150 (Austria) N: 3 (Nigeria)	Austria: BEA at 6.2 ng/L AME at 2.1 ng/L Enn B at 4.7 ng/L Enn B1, OTA, ZEN <LOQ Nigeria: AFM1, BEA, Enn B, OTA (25 ng/L), AME (65 ng/L)	-	RE±RSD: 39±19 RE±RSD: 118±18 (low spiking level)	LODs: 0.1-300 ng/L LOQs: 0.2-600 ng/L	• 1 mL • QuEChERS + SPE clean-up and enrichment step • ISTDs for SIDA • LC-MS/MS MRM (QTrap6500+)	• Braun et al., (2020) • Human mature milk • Austria

Pesticides

2.4.1b Biomarkers and matrices

Biomonitoring of pesticides is common in national programs such as the National Health and Nutrition Examination Survey (NHANES) of the United States of America (Everett & Matheson, 2010) and in epidemiological studies (Yusa et al., 2015). Blood and urine are the most commonly used matrices for measuring exposure to pesticides in human biomonitoring studies (Barr, 2008), although blood is an invasive matrix and requires trained phlebotomists. Urine on the other hand, is non-invasive, can be accessed in large volumes, which in turn allow for determination of very low concentrations of chemicals caused by environmental exposures. This is particularly relevant for currently-used organophosphate and pyrethroid pesticides (Barr, 2008). Breastmilk is also commonly monitored to measure neonatal and maternal exposures to lipophilic and persistent organic pollutants such as OC pesticides (Wasser et al., 2015). Other matrices studied include hair, and meconium.

In breastmilk, OC pesticides have been surveyed extensively since the 1970s (WHO, 2007, 2014). Despite being banned, several dominant OCs, including DDT and HCH are still seen in breastmilk samples from several countries including Croatia, India, Bangladesh, USA, Brazil, China among others (Klincic et al., 2014; Sharma et al., 2014; Bergkvist et al., 2012; Palma et al., 2014; Zhou et al., 2012). Several factors can affect levels of pesticide residues in breastmilk, including parity, duration of lactation, age, diet, habitat among others (LaKind et al., 2004; Harris et al., 2001).

Due to OC accumulation in fatty tissue, elimination occurs over a period of months to years. Most are able to cross the placental barrier and are also eliminated in breastmilk. DDE is

the primary degradant of DDT and persists in the body for longer than the parent compound. OC pesticides are found in other matrices including hair and meconium (Yusa et al., 2015).

Because non-persistent pesticides including OPs and pyrethroids are rapidly metabolized, they become excreted in urine. OPs and pyrethroids are lipophilic ($\log K_{ow} > 3$; higher the value, the more soluble a substance is in non-polar fatty substances), which allows for bioaccumulation in breastmilk (Yusa et al., 2015).

Metabolites of pyrethroid pesticides are commonly found in urine, due to their short biological half-life (hours). These metabolites are eliminated over several days after absorption. The most frequently detected metabolites of pyrethroid pesticides in urine are 3-phenoxybenzoic acid (3-PBA) and diethylthiphosphate (DETP) (Yusa et al., 2015). Urinary levels of OPs reflect recent exposure due to elimination half-life from hours to days. Urinary DAPs (dialkyl phosphates) are the most commonly used biomarkers in large population biomonitoring studies (Yusa et al., 2015).

2.4.2b Sample preparation and analytical methods

As pesticides are volatile and polar compounds, their separation in complex biological matrices involves manipulation of these properties. Several methods have been developed for extraction of pesticides from matrices. Sample preparation workflows for pesticide analysis generally include: 1) sample preparation; 2) separation; 3) detection and finally; 4) analysis of data generated. Commonly used extraction methods in pesticide analyses include liquid-liquid extraction, solid-phase extraction, matrix solid phase dispersion and QuEChERS extraction and solid phase microextraction (Samsidar et al., 2018).

The QuEChERS method is most commonly used for multiclass and multiresidue analyses, and described as quick, easy, cheap, effective, rugged and safe for sample extraction. Solid-phase extraction is also commonly used (Gonzalez-Curbelo et al., 2015).

Gas chromatography is typically used for separation of compounds using a stationary column and gas as the mobile phase, however HPLC and liquid chromatography may also be used depending on the pesticides being measured. Enzyme linked immunosorbent assays can also be used for pesticide detection (Samsidar et al., 2018). These methods collectively have high sensitivity and selectivity at low detection levels. They are complicated, laborious, costly in terms of manpower, time and instrumentation.

Several different detectors may be coupled with the GC and include mass spectrometers, triple quadrupole based tandem mass spectrometry (MS/MS) and high-resolution mass spectrometers (TOFs), electron capture detectors (ECD), flame ionization detectors (FID) among others (Martinez-Vidal et al., 2009). ECD detectors are typically used for detection of OC pesticides, whereas flame ionization detectors can be used to monitor all pesticides. OPs can be detected using various techniques including spectroscopic methods such as FTIR (Du et al., 2010); mass spectrometry (Pico et al., 2006); X-ray diffraction (Gong et al., 2012); electrochemical and NMR spectroscopy (Kumar et al., 2013).

As discussed with regards to mycotoxin analysis, mass spectrometry-based methods are superior in terms of their sensitivity, accuracy, reproducibility and ability to reduce matrix effects (Samsidar et al., 2018). Liquid chromatography can be applied for separation and determination of highly polar, non-volatile and thermally labile pesticides.

2.4.3b Comparisons of LODs, LOQs and method performance

For the purposes of this dissertation, and as described in detail in chapter 6, we used gas chromatography coupled to a mass spectrometer for detection of OC and OP pesticides in human milk samples. GC-ECD was used for detection of pesticides. Positive samples were verified using both methods. It is known that the selectivity of GC-ECD is lower than GC-MS (Liu et al., 2015). The subsequent discussion comparing existing methods for pesticide detection in human milk samples and their performance will focus on the use of GC-MS and GC-MS/MS methods developed and used internationally, and multi-residue detection studies conducted in India (between 2006-2018), to compare critical method parameters and method performance, as presented in **Table 7**, below.

As demonstrated in table 7, most pesticide analysis studies in breastmilk in the Indian context use GC-ECD, which is in line with our work. A broader discussion of comparison of LODs and LOQs and status of pesticides in breastmilk samples from India, is provided in chapter 5.

Table 7: Comparison of studies examining pesticides in animal and breastmilk from India (2006-2018)

Pesticides (classes)	Samples analyzed (N) & Positive samples (n, %)	[Range]	Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs ADIs	Extraction protocol & analytical method	Reference
HCHs and DDTs	N = 32	DDTs:0.17-0.179 mg/kg HCHs: 0.123-0.131 mg/kg	Rec: HCHs: 86.4-92.1 DDTs: 94.9-96.4 RSDs: HCHs: 6.1-8.2 DDTs: 7.4-8.0	LODs: 0.001 µg/mL LOQs: 0.01 µg/mL	• USEPA method 608 (1980) and Ejobi et al., 1996 • 20 mL milk • GC- ECD	• Kumar, Dayal, Shukla, Singh, Joseph, 2006 • Agra, India • Human milk
OCs including DDT, DDD, DDE, HCHs, dieldrin, heptachlor, endosulfan	N = 50	Milk: Rural women: 5.0625 mg/L Urban women: 3.243 mg/L Blood: Rural women: 4.608 mg/L Urban women: 3.777 mg/L	Rec: 95-98%	NR	• Takei et al., 1983 (milk) • Bush et al., 1984 (blood) • GC-ECD	• Kumar, Baroth, Soni, Bhatnagar & John, 2006 • Rajasthan • Milk and blood of women
OCPs including ΣDDT, ΣHCH, Σendosulfan, aldrin	N = 147	ΣHCH → BDL – 0.2992 12 > MRL (143 +) ΣDDT → 0.0017-0.2864 35 > MRL (147 +) Σendosulfan → BDL – 0.0317 (64 +) Aldrin → BDL – 0.1279 (17 +) ΣOCPs → 0.0039-0.5046 (147 +)	Rec: HCHs → 79-87% Endosulfans → 82-83% Aldrin → 82% DDTs → 75-81%	LODs: HCHs → 0.4-0.8 ng Endosulfans → 0.3-0.4 ng Aldrin → 0.04 ng DDTs → 0.5-0.7 ng	• Extraction: De Faubert Maunder et al., (1964) • Clean-up: Veirov and Aharonson (1978) • GC-ECD	• Sharma, Kaushik, Kaushik, 2007 • Bovine milk • Haryana

Table 7 (cont'd): Comparison of studies examining pesticides in animal and breastmilk from India (2006-2018)

Pesticides (classes)	Samples analyzed (N) & Positive samples (n, %)	[Range]	Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs ADIs	Extraction protocol & analytical method	Reference
DDTs, HCHs, PCBs, HCBs. CHLs	N = 64	• DDTs: 47-1200 ng/g lw • HCHs: 6.3-1900 ng/g lw • PCBs: 0.063-19 ng/g lw • CHLs: 0.048-21 ng/g lw • HCB: 0.4-19 ng/g lw	Rec±SD: 95±4.4%, HCH 94±5.7%, HCB 98±4.7% CHL 95±7% DDT	NR	• Kunisue et al., (2004) • 10 g milk • GC-ECD	• Devanathan et al., 2009 • New Delhi, Mumbai, Kolkata, Chennai • Human milk
HCHs, DDTs,	N = 205	ΣDDT: 2870±1570 3210±2080 ng/g lip wt ΣHCH: 2330±1160 2720±2140 ng/g lip wt	Rec: 88-97% RSD: < 12%	LOD: 0.001 ng/g LOQs: DDTs → 0.01-0.2 ng/g lipid wt HCHs → 0.02-0.3 ng/g lipid wt ADIs: DDTs: 16-18 µg/kg bw/day HCHs: 13-15 µg/kg bw/day	• Dua et al., (1997) • Defatting • Sample extracted and dried • Lipid content measured gravimetrically and concentrated sulfuric acid used to disintegrate OCPs before measurement • Clean-up using glass column • Dried extract dissolved in 2 mL hexane for GC analysis • GC-ECD	• Mishra & Sharma, 2011 • Dibrugarh and Nagaon, Assam • Human milk
HCHs, DDTs, endrin, β-endosulfan, endosulfan, sulphate, cypermethrin, chlorpyrifos,	N = 53	ΣHCHs: 199.6 ng/g lw ΣDDTs: 1914.2 ng/g lw (63% DDE in BM) Endrin: 107.64 ng/g lw β-endosulfan: 90.69 ng/g lw Endosulfan: 14 ng/g lw Cypermethrin: 45.07 ng/g lw Chlorpyrifos: 84.15 ng/g lw	Rec: 70-120%	LOD: 1 ng/g on whole milk basis → α-HCH 2 ng/g on whole milk basis→ deltamethrin	• Battu et al., (2004) • 5 mL milk • Extraction column • N-hexane:acetone (1:1) mix • GC-ECD and GC-FTD • Confirmed on GC-MS	• Bedi, Gill, Aulakh, Kaur, Sharma & Pooni, 2013 • Punjab, India • Human milk

Table 7 (cont'd): Comparison of studies examining pesticides in animal and breastmilk from India (2006-2018)

Pesticides (classes)	Samples analyzed (N) & Positive samples (n, %)	[Range]	Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs ADIs	Extraction protocol & analytical method	Reference
OCs, OPs, SPs	N = 127	HCHs: ND – 133.88 (11 > PTDI) DDE: ND-43.19 (9 > PTDI) Cyfluthrin: ND-4100.39 Cypermethrin: ND-276.35 Chorpyrifos: ND-160.48 Profenofos: ND-297.25	Rec: 65-110%	LODs: 1.0 ng/g whole milk for OCs 2.0 ng/g whole milk for OPs and SPs	• Battu et al., (2004) • 5 gm milk • GC-ECD for OCs and SPs • GC-FTD for OPs • Confirmation in GC-MS	• Sharma, Gill, Bedi, Pooni, 2014 • Punjab • Human milk
25 OCs, OPs, carbamates and pyrethroids	N = 10 each	[Median]: ND-1.365 ng/mL 19 detected in human milk	Rec in cow milk: 34-102% RSD: 1.2-12.4% Accuracy: 80-120% Precision: 0-20% Rec in baby formula: 50-80%	• LODs: 0.0033 – 1.6692 ng/mL	• ISTDs • 1 mL milk • GC-MS/MS	• Chen et al., 2014 • USA • Cow milk, human milk, baby formula
OCPs: HCHs, heptachlor, aldrin, fipronil, butachlor, dieldrin, DDTs, endrin, endosulfans SPs: cypermethrin, permethrin, cyfluthrin, deltamethrin, fenvalerate OPs: chlorpyrifos, monocrotophos, dimethoate, fenithrothion, parathion-methyl, malathion, fenamiphos, profenofos, ethion, triazophos, phosalone	N = 312	HCH: 0.9±3.5 (7% +, 12 > MRL) DDT: 1.6±3.9 (10.3% +, 1> MRL) Endosulfan: 1.2±3.8 (29%, 18 > MRL) Cypermethrin: 0.9±5 (4.1%, 1 > MRL) Chlorpyrifos: 2.2±8.5 (20%, 18 > MRL)	Rec: 81.2-113.5% RSD: <10%	LOD: 1 µg/kg – 2 µg/kg	• Battu et al., (2004) • 5 mL milk • GC-ECD for OCs and pyrethroids • GC-FTD for OPs • Confirmed using GC-MS	• Bedi et al., 2015 • Punjab • Bovine milk

Table 7 (cont'd): Comparison of studies examining pesticides in animal and breastmilk from India (2006-2018)

Pesticides (classes)	Samples analyzed (N) & Positive samples (n, %)	[Range]	Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs ADIs	Extraction protocol & analytical method	Reference
29 pesticides including OCs, OPs and SPs HCHs, DDTs, endosulfans, cypermethrin, fenvalerate, chlorpyrifos, malathion	N = 153	DDE: BDL-0.064 mg/L DDT: BDL-0.018 mg/L Chlorpyrifos: BDL-0.011 mg/L Mean ΣDDT: 0.145 mg/kg fat (26.79% +) Decade old study: 5.410 (93.04% +) Mean DDE: 0.138 mg/kg fat (26.79% +) Decade old study:4.115 (93.04% +) Mean DDT: 0.005 mg/kg fat (1.31% +) Decade old study: 1.140 mg/kg fat (1.31% +) Mean chlorpyrifos: 0.002 mg/kg fat (0.65% +)	Rec OCs and Chlorpyrifos: 91.33-120% SPs: 85.6-100%	LOQs: OCs: 0.005-0.001 mg/kg 0.01 mg/kg for chlorpyrifos 0.02-0.04 mg/kg for SPs	• 10 mL milk • QuEChERS developed • GC-ECD • Confirmed using GC-MS (selected ion monitoring)	• Sharma, Sharma, Singh, Wise, 2016 • Himachal Pradesh • Human milk

Table 7 (cont'd): Comparison of studies examining pesticides in animal and breastmilk from India (2006-2018)

Pesticides (classes)	Samples analyzed (N) & Positive samples (n, %)	[Range]	Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs ADIs	Extraction protocol & analytical method	Reference
88 different pesticides including OCs, OPs, fungicides, carbamates and pyrethroids, herbicides etc	N = 40	p,p'-DDE → month 2 79.7±72.4 (14.6-209.2) ng/g fat Month 5: 79.9±60 (19.1-188.3) ng/g fat Month 9: 51.5±52.5 (6.3-165) ng/g fat Month 12: 43±27.1 (17.5-108.5) ng/g fat	Rec: 70-120% RSD: 3.6-33.9% 2.2-17.4% 2.5-13.5% ([spiked] 10, 20, 100 µg/L)	LOD: OCP: 0.2-0.5 ng/mL LOQ: 0.5-1.0 ng/mL LOD: OPP: 0.2-1.0 ng/mL LOQ: 0.5-1.0 ng/mL LOD: fungicide: 0.2-1.0 ng/mL LOQ: 0.5-2.0 ng/mL LOD: carbamates and pyrethroids: 0.2-2.0 ng/mL LOQ: 0.5-5.0 ng/mL LOD: herbicides and other: 0.2-1.0 ng/mL LOQ: 0.5-2.0 ng/mL	• Lehotay et al., 2005, 2005 • Validated acetate buffered QuEChERs method • GC TQ triple quad mass spec • MRM	• Geddes et al., 2017 • Australia • Human milk • Longitudinal design

Table 7 (cont'd): Comparison of studies examining pesticides in animal and breastmilk from India (2006-2018)

Pesticides (classes)	Samples analyzed (N) & Positive samples (n, %)	[Range]	Calibration Curve Recoveries & Precision (RSD)	LODs, LOQs ADIs	Extraction protocol & analytical method	Reference
PCBs, DDTs, HCHs,	N = 150	Means: PCBs: 24.2-33.7 ng/g lw DDTs: 415.3-519.2 ng/g lw Total DDTs: 519.2±1017.4 (ND – 4583.1) 415.3±846.32 (ND-3212.5) HCHs: 35.5-46.6 ng/g lw Total HCHs: 46.6±106.9 (ND – 474.8) 35.5±87.3 (ND – 305.3)	Rec: 76.9-96.6% RSD: <10%	LOD: 8-20 ng/g	• Battu et al., (2004) • 10 mL milk • GC-ECD • GC-MS confirmation	• Bawa et al., 2018 • Bathinda and Ludhiana, Punjab • Day 4-6 postpartum • Human milk

2.5 Summary and research gaps

According to the Food and Agricultural Organization (FAO) of the United States, the definition of food security is "*when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life*" (Swaminathan et al., 2013). Alternative definitions of food security include "fair prices, choice, access through open and competitive markets, continuous improvements in food safety, transition to healthier diets and a more environmentally sustainable food chain" (Chakraborty & Newton, 2011). The impacts of climate change on contamination of the food supply by naturally occurring xenobiotics such as mycotoxins, which are a global problem seen in weather-damaged grains or grains harvested with high moisture content (Blaney et al., 1987), have severe consequences for human and animal health (Haschek et al., 2002). The excessive use or misuse of chemical xenobiotics including pesticides to ensure adequate food security both locally and globally, is also known to cause serious and long-lasting adverse effects on the health of the ecosystem, animals and at the top of the food chain, human beings (Nicolopoulous-Stamati et al., 2016). Many unanswered questions remain about the extent of xenobiotic exposures in human breastmilk and associated impacts on infant growth and development. The studies presented in this dissertation, although small in scale aim to understand the presence of mycotoxins and pesticides in human breastmilk samples from the north Indian state of Haryana, and their potential associated risks to the health of mothers and infants.

NOTE: Reference list for Chapter 2, 7 and 8 are located at the end of this dissertation

Chapter 3: Quantification of aflatoxin and ochratoxin contamination in animal milk using UPHPLC-MS/SRM method: a small-scale study

Short Title: Mycotoxins quantification in milk

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Research Highlights:

- Method developed and validated to quantify six aflatoxins and two ochratoxins in raw animal milk samples
- Aflatoxin B_2, Aflatoxin M_1 and Aflatoxin M_2 were detected in cow, goat and pasteurized cow milk samples but not in buffalo milk
- 90% of cow, goat and pasteurized milk samples were over the EU limits (50 pg/mL) for Aflatoxin M_1
- 40% of cow and goat milk samples were over FSSAI limits (500 pg/mL) for Aflatoxin M_1

Key words: Aflatoxin, Ochratoxin, Milk, UHPLC-MS/SRM method, Quantification.

Abstract

Mycotoxin contamination in animal milk is an emerging concern around the globe. Here we developed and validated an ultra-high-performance liquid chromatography tandem mass spectrometry-selected reaction monitoring (UHPLC/MS-SRM) method to quantify low concentrations of aflatoxins (AFs) and ochratoxins (OTs) in routinely consumed animal milk samples collected from southern India. Stable isotope dilution was applied to quantify AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2 and OTA, OTB in n = 38 different milk samples, using 1 mL of milk. Bioanalytical parameters including method accuracy, precision, recovery, regression analysis and stability were assessed. Dynamic ranges for quantification were between 15.6-1000 pg/mL for AFB_1, AFB_2, AFG_1, and OTA; 7.8-500 pg/mL for AFM_1, AFM_2 and OTB; 78.6-5000 pg/mL for AFG_2. Method accuracy ranged between 80-120%, with ± 15% precision. Recoveries for spiked standards were >88% in water and 75% in milk, with limits of quantification ranging between 31.3 pg/mL for AFB_1, AFB_2, AFG_1 and OTA, 15.6 pg/mL for AFM_1, AFM_2 and OTB and 156 pg/mL for AFG_2. R^2 values for regression analyses ranged between 0.9991-0.9999. AFB_2 [mean: 38 pg/mL (0.038 µg/kg)] was quantified in goat milk, AFM_1 was quantified in cow, goat, pasteurized milk [mean: 331 pg/mL (0.331 µg/kg), 406 pg/mL (0.406 µg/kg), 164 pg/mL (0.164 µg/kg)]. Additionally, 90% of cow, goat and pasteurized milk samples were above European Union (EU) limits of 50 pg/mL (0.05 µg/kg) and 40% of goat milk samples were above the Food Safety Standards Authority of India (FSSAI) limit of 500 pg/mL (0.5 µg/kg). AFM_2 was also quantified in cow, goat, and pasteurized milk samples [mean: 249 pg/mL (0.249 µg/kg), 375 pg/mL (0.375 µg/kg), 81 pg/mL (0.081 µg/kg)]. Our dynamic ranges for quantification are lower than other published methods, with need for a smaller volume of milk. This validated method can be applied for routine detection of mycotoxins in milk.

1. INTRODUCTION

Mycotoxin contamination in food items is an issue of growing concern around the globe, especially in countries like India, due to the emerging effects of climate change (Bhat et al. 2010). These toxins account for millions of dollars' worth of losses in agricultural products and result in adverse impacts on human and animal health (Alshannaq et al. 2017). The Food and Agriculture Organization estimates that 25% of the global food system is impacted by mycotoxin contamination. Several factors contribute to the presence or production of mycotoxins in foods and feed, including storage, environmental and ecological conditions (Hussein et al. 2001). In case of animal source foods, contamination happens primarily due to fungal growth in animal feed. Human exposure to mycotoxins occurs via intake of contaminated agricultural products such as cereals, corn and indirectly via the consumption of animal source foods such as milk and eggs (Flores-Flores et al. 2015). Tackling fungal growth that leads to mycotoxin/secondary metabolite contamination in food and feed is challenging due to their presence in a wide range of crops (Munkvold 2003; CAST. 2003).

Secondary fungal metabolites produced by *Aspergillus, Penicillium* and *Fusarium* genera, that are known to contaminate crops and animal feed lead to a wide range of adverse health effects (Ismaiel et al. 2015). Aflatoxins (AFs), which are produced by *Aspergillus parasiticus* and *Aspergillus flavus* are primarily found in hot and humid climates (Giorni et al. 2007; Passone et al. 2010). Human exposure to AFs occurs mainly via consumption of staple crops such as maize and groundnuts, which have been impacted by *A.flavus* and/or *A.parasiticus*, in addition to foods derived from animals. AFB_1 is the most toxic of the AFs and chronic exposure is known to cause liver cancer and several other adverse health outcomes in humans. It has been categorized as a Group 1 carcinogen by the International Agency for Research on

Cancer (IARC) (Ostry et al. 2017). AFB_1 is metabolized into an epoxide form that results in adduct formation with DNA and albumin through lysine modification (Sabbioni et al. 2017; Woo et al. 2011). Hepatic hydroxylation of AFB_1 by CYP1A2 forms AFM_1, which is excreted into the milk of lactating animals and is considered an important marker of long-term AFB_1 exposure (Sabbioni et al. 2017; Woo et al. 2011). AFM_1 is tenfold less toxic than AFB_1 and is classified as a group 2B carcinogen (probable carcinogen) by the IARC (Ostry et al. 2017).

Ochratoxins are produced by fungi of genera *Aspergillus* and *Penicillium*, commonly found in foodstuffs and feed (Zhu et al. 2017). OTA has been categorized as a group 2B carcinogen by the IARC (Ostry et al. 2017). Dietary exposure to OTA represents an important concern to public health and has been associated with several diseases in humans and animals (Heussner et al. 2015; Pfohl-Leszkowicz. 2009; Stoev et al. 2013). Several naturally occurring conjugates and metabolites of OTs have been identified to date, however the main forms include OTA, OTB, OTC and OTα (Malir et al. 2016). OTB is a non-chlorinated form of OTA and OTC is an ethyl ester of OTA.

A variety of methods exist for detection of mycotoxins in different food matrices and animal feed including HPLC based fluorescence detection (HPLC-FD) (Kumar et al., 2020; Dhanshetty et al., 2019; Oulkar et al., 2017) and enzyme linked immunosorbent assays (ELISA) (Pestka et al. 1981). These methods have lower sensitivity and assays require higher sample volume. Due to the limitations in these methods, liquid chromatography-mass spectrometry-based quantification using stable isotope labelled internal standards is the current gold standard method in the field, for detection and quantification of lower levels of multiple-mycotoxins (pg to ng ranges) in food matrices.

Several published papers have described different kinds of mass spectrometry-based methods for analysis of mycotoxins, but have either not used labelled internal standards (ISTDs) or quantified AFM_2 (Mao et al. 2018; Zhang et al. 2018). We therefore developed and validated a multi-analyte method to quantify aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2) and ochratoxins (OTA and OTB) from a variety of milk samples (cow, goat, buffalo and pasteurized milk) by using labelled AFB_1-D_3 and OTA-D_5, as internal standards.

2. EXPERIMENTAL

Materials

Mycotoxin standards for AFB_1, AFB_2, AFG_1 and AFG_2 were purchased from Sigma-Aldrich (Bangalore, India). Standards for AFM_1, AFM_2, OTA, OTB, AFB_1-D3 and OTA-D5 were procured from Toronto Research Chemicals (Toronto, Canada). The purity of all of the analytes and deuterated internal standards was ≥98%. High purity MS grade solvents (water and acetonitrile) were purchased from Honeywell (Bangalore, India). Formic acid, ammonium acetate were obtained from Sigma-Aldrich (Bangalore, India). AFB_1, AFB_2, AFG_1 and AFG_2 were purchased as 1 mg; AFM_1 as 50 µg; AFM_2 as 100 µg; OTA/OTB as 2 mg in powder form. AFB_1-D3 and OTA-D5 ISTDs were purchased as 0.25 mg and 0.5 mg in powder form, respectively. These STDs and ISTDs were dissolved in acetonitrile to make 100, 10 µg/mL stocks and stored at -80 °C. Centrifugal membrane filters (0.45 µm, PVDF) were obtained from Thermo-Fisher Scientific (Bangalore, India). Animal milk (from cow, goat and buffalo) and pasteurized cow milk sample collection and storage conditions are presented elsewhere (Shetty et al. 2020).

Standard stock preparation

Working stock solutions for all mycotoxin standards, two internal standards and serial dilutions were prepared in acetonitrile. Working stock concentrations ranged from 1 μg/mL for AFB_1, AFB_2, AFG_1, OTA and OTB, 0.5 μg/mL for AFM_1 and AFM_2 and 10 μg/mL for AFG_2. For both AFB_1-D3 and OTA-D5 the working stock concentrations were 1 μg/mL.

3. UHPLC-MS

A Sciex QTRAP 6500 (Sciex Singapore) mass spectrometer was used for mycotoxin analysis, which is equipped with a turbo V ion source for effective ionisation. The mass spectrometer is coupled to an Agilent 1290 infinity II UHPLC system (Agilent Technologies India Pvt. Ltd., India), and equipped with a column oven (set at 40 °C), auto-sampler with a thermo-controller (set at 10 °C). The system uses a flow through needle mode after injection and is furnished with a needle wash system (with 50% methanol) before injection to ensure zero percent carry over problems. The mobile phase solvent A was water (10 mM Ammonium Acetate, 0.1 % Formic Acid) and Solvent B was acetonitrile (0.1% Formic Acid). In the UHPLC system, a C-18 column (2.1 × 100 mm, 1.8 μm, Agilent, Inc) was used for separation of mycotoxins. An optimised gradient to get maximum separation (0 to 3 min-10%B, 3 to 15 min-10%B to 80% B, 15.1 to 17 min-100% B, 17.1 to 22 min-10%B) at 200 μL/min flowrate was used for analysis. The injection volume (10 μL) was kept constant throughout the analysis. Operating conditions were: Spray Voltage- 5500 V, Curtain Gas- 30 psi, Temperature- 500 °C, Gas 1- 30 psi, Gas 2- 50 psi, Entrance Potential (EP)- 10 V. Other parameters including De-clustering Potential (DP), Exit Potential (CXP), and Collision Energy (CE) were standardised based on the compound and incorporated into the method. Scan time was 50 millisec per

transition with positive ion polarity. To obtain the details of tandem mass spectrometry (MS/MS) scans, a syringe pump was used to infuse STDs with flow of 10 μL/min. For each metabolite, the precursor ion was monitored and collision induced dissociation was used to generate product ions. The product ions were obtained by scanning the quadrupole-3 from m/z 50 to 500 with a cycle time of 1 sec. This was followed by optimization of DP, CXP and CE for each of the intense product ions as shown in Table 1.

Calibration curves

Calibration curves were set in the range of 15.6-1000 pg/mL for AFB_1, AFB_2 and OTA. For AFM_1, AFM_2 and OTB, the range was set to 7.8-500 pg/mL and for AFG_2 the range was higher at 78-5000 pg/mL. Internal standard (ISTD) mix (AFB_1-D_3 and OTA-D_5) was spiked in concentration of 1000 pg/mL to each standard. To extract mycotoxins from 1 mL of milk samples, 2 mL of cold acetonitrile with 2% formic acid was used. Similarly, to construct the standard curves, both STDs and ISTDs were spiked into 3 mL of water:acetonitrile (2% FA) (1:2) solution and processed similarly. Briefly, the STDs and ISTDs were extracted by separating the acetonitrile layer using a concentrated salt solution [300 μL of concentrated ammonium acetate (10 g/10 mL)]. The mixture was sonicated in a water bath sonicator for 10 min; it was subsequently removed and kept on ice for 5 min. It was then centrifuged for 5 min, (3800 x g) to separate the acetonitrile layer. Approximately, 1.5 mL of the acetonitrile from the supernatant was transferred to a 2 mL Eppendorf tube and dried (2.5 hours in speed vacuum). The dried extract was then reconstituted with 100 μL of 50% acetonitrile (vortexed and centrifuged for 5 min at 15000 x g) and 80 μL was transferred to HPLC vials. Next, 10 μL was injected to analyse mycotoxins using the UHPLC-MS/SRM method. STDs were prepared to construct a seven-point

calibration curve on a daily basis, and analysed along with five replicates of quality control (QCs) samples as shown in table 2.

Method Validation

The most important bio-analytical parameters for validation were conducted using criteria established by the United States Food and Drug Administration (US FDA) (Guidelines for Bioanalytical Method Validation. 2018). Calibration linearity was studied by spiking two internal standards (one for each group of mycotoxins: AFB_1-D3 for AFs and OTA-D5 for OTs) to calibration solution at seven concentrations for mycotoxins. Previous studies have demonstrated the use of a single chemically similar ISTD for quantification of compounds in complex matrices, without a compromise in assay performance (Wieling. 2002).

Integrated peak areas of the selected SRM transitions were used to build the STD curves. The highest intense product ion from each mycotoxin was used to build the STD curve. Curves were fitted by an equal weighted regression analysis using the quantification software Analyst (version 1.6.3). Precision and accuracy were evaluated using four concentration points (Limit of Quantification-LOQ; Lower Quality Control-LQC; Middle Quality Control-MQC and High-Quality Control-HQC). Accuracies of 85–115% and precisions of ±15% were considered acceptable for LQC, MQC and HQC samples. Accuracies of 80-120% and precisions of ±15% were considered acceptable for the LOQ as recommended. Five replicates for each point were analyzed to determine the intra and inter-day accuracy and precision. This process was repeated over 3 days in order to determine the inter-day accuracy and precision using freshly prepared calibration curves. Accuracy was determined by the recovery of QC, and the precision was expressed as the coefficient of variation (CV) of the determination of the QCs. Inter-day

accuracy and precision were calculated similarly for 15 replicates of each concentration point pooled from the three validation runs.

Recovery, Stability and Matrix effect

To check recovery from the milk matrix, triplicate QCs (LQC, MQC, HQC) and ISTDs were spiked to milk: acetonitrile (1:2) and water:acetonitrile (1:2) solvent system. As a control, both milk and water alone without the STDs were used. The STD curve was constructed in the same way to calculate the recovery from both milk and water matrices. The acetonitrile phase separation was done in the same way as mentioned above. The solvent was evaporated in a speed vacuum and reconstituted in 100 μL of 50% acetonitrile and 10 μL was injected for the analysis. Freeze thaw stability was checked by spiking QCs in triplicate alongside ISTDs to milk, which was allowed to freeze in the -80 °C for 20 min. We repeated the freeze thaw cycle thrice and calculated the concentration of the spiked toxins by using the STD curve. The stability in the auto-sampler vial was checked after 24 hrs for the highest standard.

To check for matrix effects, 12 aliquots of control pooled milk samples were processed in the same way as described above and the supernatant was then dried completely. Three samples were used as controls and in the remaining nine samples, triplicate QCs were spiked (n = 3 of each, LQC, MQC and HQC) on top of the processed milk matrix and reconstituted to 100 μL of 50% acetonitrile and 10 μL was injected for analysis. As a control, QCs were spiked directly to reconstituted solution (100 μL of 50% acetonitrile) and 10 μL was injected for analysis. These two datasets were compared to calculate matrix effect.

Sample preparation from different milk samples

The aliquoted milk samples (1 mL) were allowed to thaw on top of ice and transferred to 15 mL centrifuge tubes. Ice-cold acetonitrile (2 mL) with 2% formic acid was then added, and 10 μL of ISTDs were spiked on top. The sample was then vortexed for 1 min and kept on ice for 5 min to allow for complete protein precipitation. Following this, 300 μL of concentrated ammonium acetate (10 g/mL) solution was added, sonicated in a water bath sonicator (10 min), kept on ice (5 min), and then centrifuged (5 min, 3800 x g) to separate the acetonitrile layer. Approximately 1.5 mL of the acetonitrile from the supernatant was transferred to a 2 mL Eppendorf tube and dried in a speed vacuum. The final reconstitution was done with 100 μL of 50% acetonitrile and filtered through a 0.45 μm PVDF membrane centrifugal filter (5 min, 15000 x g). The top 80 μL from each sample was transferred to HPLC vials and 10 μL from each were injected to analyse mycotoxins in milk by UHPLC-MS/SRM method. A seven-point calibration curve was constructed and pooled cow milk samples and solvent alone controls were used throughout the study for quantification of mycotoxins.

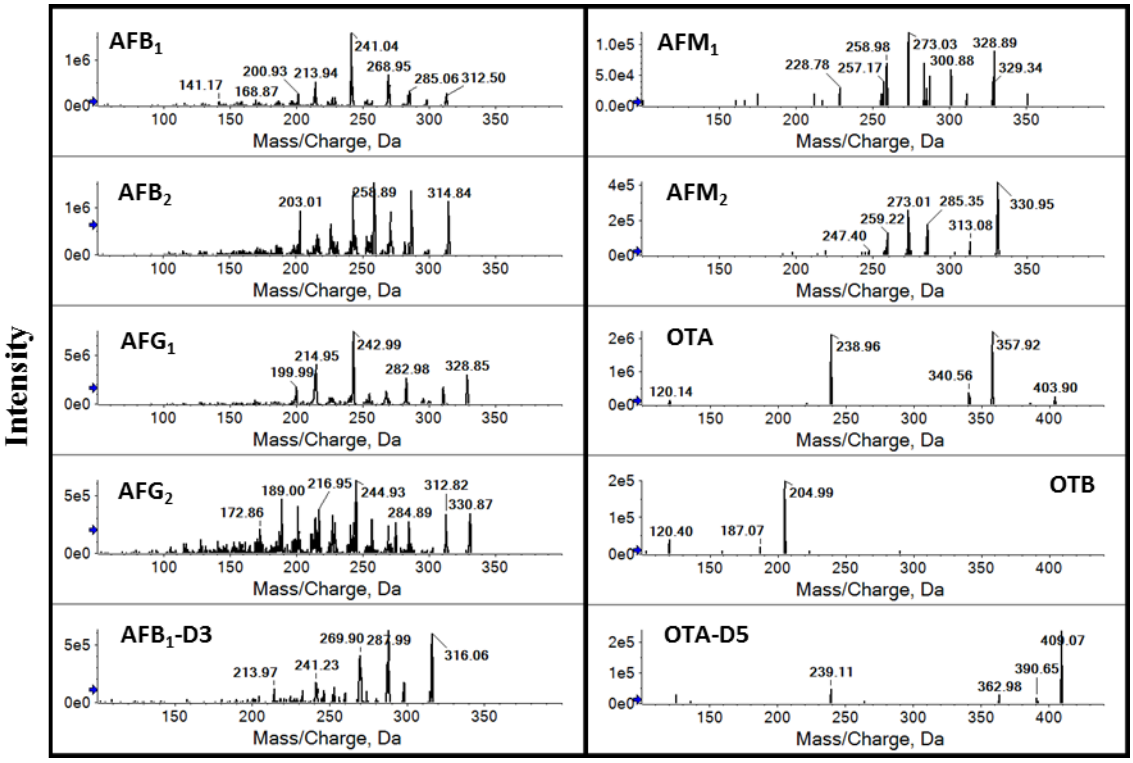
4. RESULTS

UHPLC-MS/MS analysis of mycotoxins

Infusion of 10 μg/mL solution of each mycotoxin in acetonitrile was conducted in the mass spectrometer through the syringe pump at a flow rate of 10 μL/min. The source temperature was kept at 0 °C and the other parameters including curtain gas-20 psi, gas 1-10 psi were set to check the full MS and the MS/MS details. Both AFs and OTs showed the M+H ion as major parent ion in the mass spectrum (Supplementary figure 1). The collision induced dissociation of the M+H ions showed the product ions of which the highest intense one was selected for

quantification. AFB_1 & B_2, AFG_1 & G_2 and AFM_1 & M_2 showed similar patterns of product ions (**Figure 1**).

Figure 1: MS/MS analysis of aflatoxins, ochratoxins and the corresponding internal standards.



In case of OTA and OTB the highest intense product ion is similar with and without a chlorine moiety (m/z: 239 for OTA and 205 for OTB). The highest intense product ions were selected to make the SRM method in the MS (**Table 1**). AFB_1-D3 and OTA-D5 showed the highest intense product ions, which are similar to AFB_1 and OTA indicating the common un-labelled product ions.

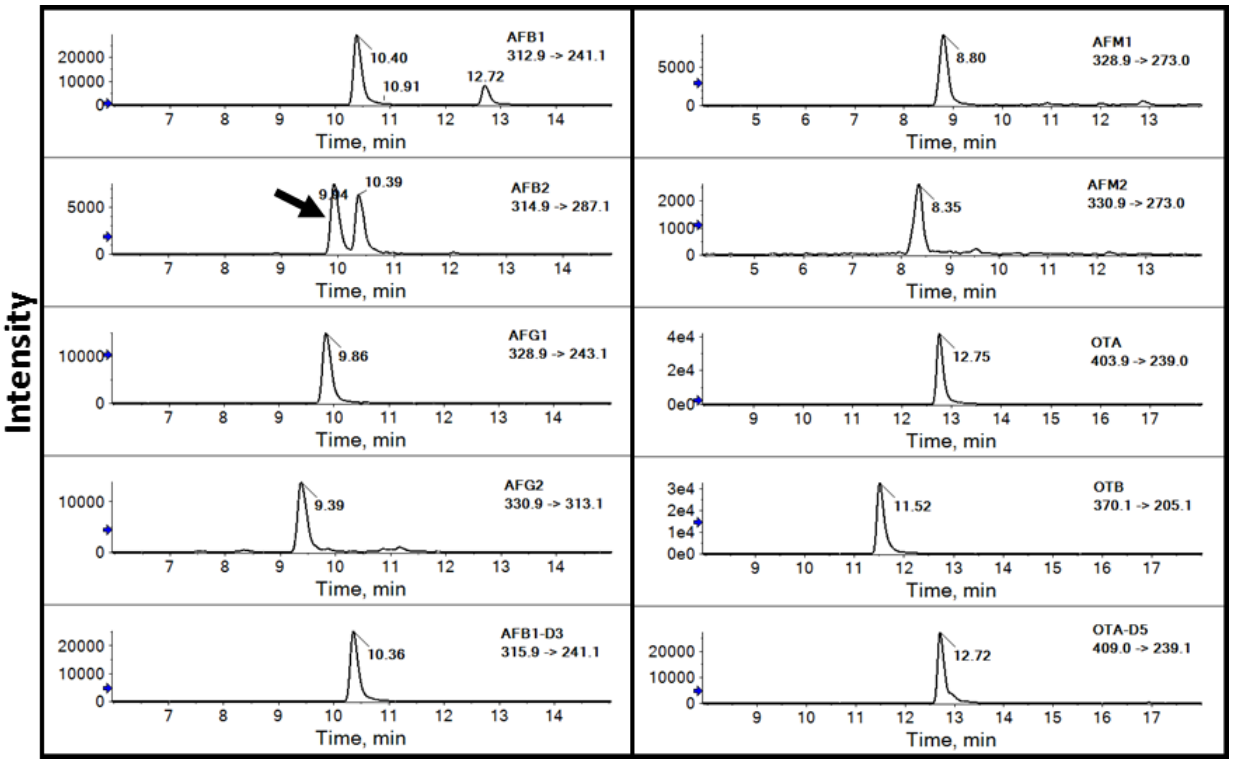
Table 1: Selected Reaction Monitoring Table

Analyte	Parent ion (m/z)	Product ion (m/z)	Retention Time (min)	Declustering Potential (volts)	Collision Energy (CE)	CXP (volts)	Equation	R^2
AFB_1	312.93	241.08	10.4	161	53	30	y = 0.0117x + 0.0138	0.9995
AFB_2	314.95	287.08	9.9	176	39	26	y = 0.0039x - 0.001	0.9995
AFG_1	328.92	243.05	9.9	161	39	22	y = 0.0076x - 0.0015	0.9999
AFG_2	330.96	313.13	9.4	131	35	32	y = 0.0018x + 0.0047	0.9998
AFM_1	328.96	273.04	8.8	171	33	38	y = 0.0092x + 0.0009	0.9998
AFM_2	330.92	273.05	8.4	146	33	32	y = 0.0038x - 0.0003	0.9999
AFB_1-D_3	315.95	241.09	10.3	186	33	28		
OTA	403.94	239.02	12.7	61	21	20	y = 0.0099x - 0.0056	0.9963
OTB	370.06	205.06	11.5	76	27	22	y = 0.0231x - 0.0102	0.9935
OTA-D_5	409.02	239.05	12.7	16	33	32		

Method development

Pooled cow milk samples were initially checked for use as matrix matched controls during method development. However, AFM_1 and AFM_2 were present in quantifiable amounts, which made it difficult to use milk as a control matrix for method validation. We therefore used the same amount of extraction solvent (1:2 of water and acetonitrile) with 2% FA as matrix for method development and validation. Briefly, both STDs and ISTDs were spiked to 3 mL of extraction solvent and the acetonitrile layer was separated, dried, and reconstituted in 50% acetonitrile and injected. Both AFs and OTs showed clear peaks in the UHPLC-MS/SRM chromatogram (**Figure 2**).

Figure 2: UHPLC-MS/SRM chromatogram of Standards and Internal Standards in HQC level



[a]The arrow indicates the peak for AFB_2 standard.

Standard curves were constructed using AFB_1-D3 as ISTDs for all AFs and OTA-D5 for both OTs in order to quantify milk samples. There is a 0.4 min difference in the retention time between AFB_1 to B_2, AFG_1 to G_2 and AFM_1 to M_2. In the case of OTs, there is a 1.3 min retention difference between OTA and OTB. Another peak in the AFB_2 channel, which is close to its retention time, is associated with AFB_1-D3 ISTD. The internal standard showed a major peak at 316 m/z and a minor peak at 315 m/z in the AFB_2 channel. Since there is a 0.4 min difference in retention time, the peak does not cause interference with quantification of AFB_2. Both AFs and OTs elute between 8 and 13 minutes in the gradient (30-70%B) in the analytical column. All mycotoxins were stable in the auto-sampler for at least 24 hours at 10 °C. In order to assess recovery, QCs (LQC, MQC, and HQC) were spiked to both milk and water matrices, following the same procedure described above. Recovery was calculated based on the

concentration of mycotoxins in milk and the amount spiked on top of milk. Under these conditions the recovery for all toxins were above 88% when spiked into water and above 75% when spiked into pooled milk (Supplementary Table 1). Recovery calculations were made based on controls using both water and milk alone. Matrix effects did not appear to be of concern and recoveries were above 85% for MQC and HQC. At the LQC level, both OTA and AFM_2 showed 30% reduction in concentration compared to the concentration spiked (Supplementary Table 2). As noted by Panuwet et al., (2016), the use of isotopically labelled internal standards can minimize the impact of biological matrix effects on quantification, although there remains a lack of definitive consensus on management of such effects during bioanalytical method development and validation (Panuwet et al. 2016).

Calibration curve and limit of quantification

Calibration curves for quantification of each of the mycotoxins were linear over a seven-point standard curve range spanning 64-fold, with regression correlation coefficients ranging between 0.9991 and 0.9999 (Supplementary figure 2). Standard curves for AFB_1, B_2, G_1 and OTA ranged between 15.6 pg/mL to 1 ng/mL; for AFM_1, M_2 and OTB ranged between 7.8-500 pg/mL and for AFG_2 ranged between 78 pg/mL to 5 ng/mL. Regression analyses were conducted for calibration curves with the mean (±SD) for the slope and intercept on three different days (**Table 1**). The LOQs were defined as the lowest analyte concentration that could be quantified with an accuracy of 80-120% and precision of ± 15% for replicates (n=5) on three different days, with a corresponding signal to noise ratio > 10 (**Table 2**). The ranges for each mycotoxin were selected based on detected response in the mass spectrometer.

Table 2: Method validation of Aflatoxins and Ochratoxins

	LOQ	LQC	MQC	HQC	LOQ	LQC	MQC	HQC
	AFB1				AFB2			
Concentration in pg/ml	31.30	62.50	250.00	800.00	31.30	62.50	250.00	800.00
Inter-day mean (n = 3)	31.08	61.77	253.13	809.53	30.05	61.39	249.13	807.58
%CV (n = 3)	5.91	5.52	3.23	3.34	3.73	5.75	5.04	0.90
Accuracy (%)	99.30	98.84	101.25	101.19	96.00	98.23	99.65	100.95
	AFG1				AFG2			
Concentration in pg/ml	31.30	62.50	250.00	800.00	156.00	313.00	1250.00	4000.00
Inter-day mean (n = 3)	31.53	63.74	253.27	793.57	158.60	315.87	1244.67	4019.50
%CV (n = 3)	2.76	1.15	0.66	2.86	0.67	3.71	4.78	0.36
Accuracy (%)	100.75	101.98	101.31	99.20	101.67	100.92	99.57	100.49
	AFM1				AFM2			
Concentration in pg/ml	15.60	31.30	125.00	400.00	15.60	31.30	125.00	400.00
Inter-day mean (n = 3)	15.52	31.03	123.93	396.73	15.40	30.69	125.13	400.80
%CV (n = 3)	3.80	3.67	4.06	4.12	3.90	5.25	3.47	2.05
Accuracy (%)	99.49	99.15	99.15	99.18	98.72	98.04	100.11	100.20
	OTA				OTB			
Concentration in pg/ml	31.30	62.50	250.00	800.00	15.60	31.30	125.00	400.00
Inter-day mean (n = 3)	31.46	61.80	253.20	815.55	15.36	31.27	127.73	409.23
%CV (n = 3)	3.87	2.16	3.09	1.41	5.88	2.65	1.49	0.94
Accuracy (%)	100.51	98.88	101.28	101.94	98.46	99.91	102.19	102.31

[a]LOQ = limit of quantification; LQC = lower quality control; MQC = middle quality control; HQC = higher quality control

Assay accuracy, precision and stability

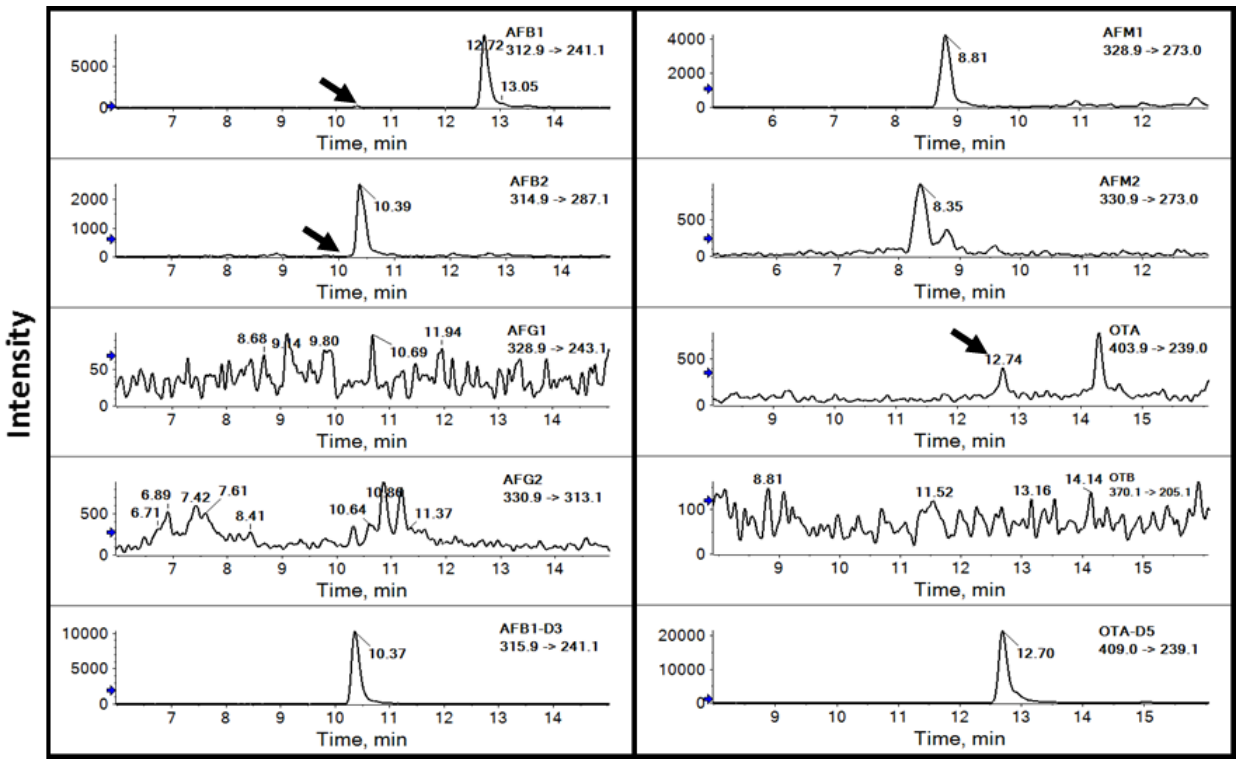
Overall, excellent accuracy and precision were obtained for the analysis of all mycotoxins (**Table 2**). Inter-day accuracy (n = 3) for the LQC (62.5 pg/mL for AFB_1, AFB_2, AFG_1 and OTA; 31.3 pg/mL for AFM_1, AFM_2 and OTB; 313 pg/mL for AFG_2) for all toxins ranged from 98 to 102%. For MQC (250 pg/mL for AFB_1, AFB_2, AFG_1 and OTA; 125 pg/mL for AFM_1, AFM_2 and OTB; 1250 pg/mL for AFG_2) from 99 to 102%. For HQC (800 pg/mL for AFB_1, AFB_2, AFG_1 and OTA; 400 pg/mL for AFM_1, AFM_2 and OTB; 4000 pg/mL for AFG_2) from 99 to 102%. Inter-day precisions (n = 3) were in the range of 1-6% (for LQC), 1–5% (for MQC) and 1–4% (for HQC). The typical STD curves for all AFs and OTs are shown in the supplementary figure 2. Lines were constructed using ratios (Analyte/ISTD) against the concentrations and passed close to the point of origin with regression ranges between 0.999 to 1.

Details of the method development data are tabulated in **table 2**. The precision and accuracy data for the analysis of the LOQ, LQC, MQC, and HQC samples that were re-analysed after 24 hours standing on the auto sampler gave essentially identical data to that obtained from the original analyses.

Analysis of mycotoxins from different milk samples

Mycotoxins from different milk samples (cow, goat, buffalo and pasteurized milk samples) were processed and analysed using the validated UHPLC-MS/SRM method. AFG_1, G_2 and OTB showed no peaks, AFB_1 and B_2 showed slight humps at the corresponding retention times and AFM_1, M_2 and OTA showed clear peaks in cow milk samples. The typical UHPLC-MS/SRM chromatogram of one of the cow milk samples is shown in **figure 3**.

Figure 3: UHPLC-MS/SRM chromatogram of cow milk sample

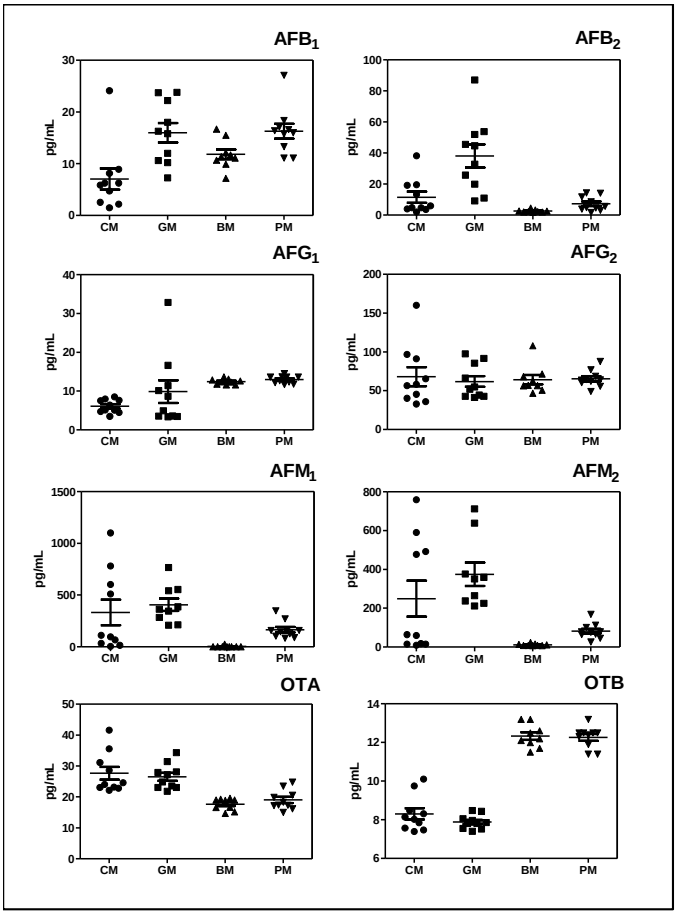


[a]The arrow indicates the peak for AFB_1, AFB_2 and OTA.

A small hump was seen in the AFB_1 and AFB_2 channels at the exact retention times in the chromatogram. A background peak was seen at 12.72 min in the AFB_1 channel, which was away from the AFB_1 peak. This validated method allows for detection of even trace levels (~10 pg/mL) of these toxins in animal milk samples. Concentrations of AFB_1 in all milk samples were below the LOQ level (31.3 pg/mL). One of the cow's milk samples, one pasteurized milk and five goat milk samples were above the first point (detection limit) of the standard curve (15.6 pg/mL). Buffalo milk samples were below the limit of detection for AFB_1. For AFB_2, both buffalo and pasteurized milk samples were below the first point of the STD curve (15.6 pg/mL). Five goat milk samples and one cow's milk sample showed levels above the LOQ (31.3 pg/mL) for AFB_2. Mean levels of AFB_2 in goat milk samples were 38 pg/mL (0.038 μg/kg). In case of AFG_1, all milk samples were below the first point and LOQ except one sample, which showed ~33 pg/mL (~0.033 μg/kg). For AFG_2, all milk samples were below the LOQ except one cow's milk sample, which showed ~160 pg/mL (~0.16 μg/kg). In case of AFM_1/AFM_2, with the exception of buffalo milk, all other samples showed quantifiable amounts. Seven of the fresh cow's milk samples and all goat/commercial milk samples showed levels that were higher than the permissible limits for AFM_1, which is 50 pg/mL (according to the EU). Similar patterns were seen for AFM_2 in milk samples. Four cow milk samples and three goat milk samples showed concentrations above the allowable limits (500 pg/mL according to FSSAI and CODEX). Mean values for AFM_1 in cow milk and in goat milk samples were 331, 406 pg/mL (0.331, 0.406 μg/kg) and for AFM_2 in cow milk and in goat milk samples were 249, 375 pg/mL (0.249, 0.375 μg/kg). OTA concentrations were less than the LOQ value but were above the limit of detection (15.6 pg/mL) for cow and goat milk (22 pg/mL or 0.022 μg/kg). Interestingly, both buffalo and pasteurized milk samples showed levels of OTB ~12 pg/mL (0.012 μg/kg) that were higher when

compared to cow and goat milk samples which showed ~8 pg/mL (0.008 µg/kg). Both were slightly above the limit of detection (7.8 pg/mL). A complete analysis of AFs and OTs from a variety of milk samples is shown in **figure 4.**

Figure 4: Analysis of Mycotoxins in milk



[a]Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2), ochratoxins (OTA and OTB)
[b]CM-cow milk, GM-goat milk, BM-buffalo milk, PM-Pasteurized milk

5. DISCUSSION

India is the leading producer of liquid milk, accounting for 17% of the world's total milk production (Douphrate et al. 2013). Milk, particularly from dairy animals such as cows, goats and buffalo provide essential micro and macronutrients, which help children and adults meet their daily energy and nutrient needs. It is estimated that on average 1 glass of milk (250 mL) per day, is consumed by the general populace in India, either directly or indirectly in a variety of

forms such as tea, coffee, curd, butter milk etc., (Kumar et al. 2014). Pasteurized or ultra-high temperature treated milk is consumed in urban areas across the country. In rural regions, however fresh milk is collected directly from animals and distributed to households on a daily basis. High levels of milk consumption make it imperative to assess contamination and adulteration, routinely. A recent National Milk Quality Survey (2018) conducted by the Food Safety and Standards Authority of India (FSSAI) collected samples from 29 states and 7 union territories across the country and found that 5.7% of milk samples were contaminated with AFM_1 (FSSAI. 2018). LC-MS/MS methods were used for quantification of AFM_1 in milk samples, however standard curve ranges were lower in our analysis, thus allowing for detection of pg/mL levels of exposure. Previous studies on animal milk have reported AFM_1 in samples from across the globe at levels in the range of 0.3-1718 pg/mL, thus higher method sensitivity can improve detection of even trace concentrations of exposures in complex matrices such as milk (Ketney et al. 2017).

During the method development process, we checked different sample preparation protocols including direct protein precipitation (using ethanol, methanol and acetonitrile), liquid-liquid extraction (LLE) with hexane after protein precipitation and reverse phase solid phase extraction (RP-SPE) after protein precipitation for mycotoxin extraction from milk. We observed poor recoveries under all the aforementioned conditions (data not shown) and finally used ice-cold acidic acetonitrile for mycotoxin extraction. To separate the acetonitrile layer, concentrated ammonium acetate solution was used instead of sodium acetate, magnesium sulphate and sodium chloride. This is mainly to avoid issues with ionization in the mass spectrometer. Better extraction efficiency with minimal steps in sample preparation (protein precipitation, acetonitrile

phase separation, drying the acetonitrile layer and analysis) may explain our ability to detect and quantify lower concentrations (pg/mL) of AFM_1/M_2 and AFB_2 in milk samples.

To assess mycotoxin contamination in milk, we collected fresh cow, goat and buffalo milk samples (n = 28). We also collected pasteurized cow milk samples (n =10), to see if there are differences in mycotoxin levels due to the effects of ultra-high temperature processing. With the exception of three cow milk samples, all other animal milk samples (cow, goat and pasteurized) showed AFM_1 above 50 pg/mL, which exceed maximum allowable limits set by the EU. Four of the goat milk samples, exceeded the FSSAI and CODEX maximum allowable level (MAL) of 500 pg/mL. Interestingly, none of the buffalo milk samples showed AFM_1 contamination. Buffalo milk samples were acquired from a hilly and elevated part of southern India, where an improved grazing environment may explain lower levels of mycotoxin contamination in animal feed.

We found that AFM_2, a derivative of AFB_2, is also present at higher concentrations (mean of AFM_2 in cow milk: 249 pg/mL (0.249 μg/kg), goat milk: 375 pg/mL (0.375 μg/kg), buffalo milk: 11 pg/mL (0.011 μg/kg) and pasteurized milk: 81 pg/mL (0.081 μg/kg). Four of the cow milk samples and two of the goat milk samples exceed 500 pg/mL (0.5 μg/kg) limit for AFM_2. The biological implications of AFM_2 contamination on health have not been well characterized. Further research is needed to understand the synergistic or additive effects of AFM_1 and AFM_2 on health. Levels of AFB_1 and AFB_2 in milk were much lower when compared to AFM_1 and AFM_2, which suggests that AFB_1 and AFB_2 are metabolized and secreted into milk in the form of AFM_1/AFM_2. Levels of AFG_1 and AFG_2 are below LOD in most of the milk samples. Studies conducted globally show that approximately 9.8% of milk samples exceed maximum limits set for AFM_1 whereas other mycotoxins are found at lower concentrations in milk (Milicevic et al.

2015). There is a need for more systematic investigation of the levels of aflatoxins in milk used for regular human intake, particularly in countries with high dairy consumption, such as India. In case of Ochratoxins, most of the milk samples were above the first point in the STD curve for OTA and OTB, but were below the LOQ. OTA has been previously reported in milk, infant formula and milk-based products (Malir et al. 2016).

Quantification of lower pg levels of mycotoxins in food matrices, using smaller volumes of matrix is a major challenge. LC-MS offers the best approach for quantification of metabolites, nutrients and other contaminants in food matrices. Due to higher method sensitivity, selectivity and specificity, UHPLC-MS/SRM is considered the gold standard method for quantification of biomolecules, when compared to UV, fluorescence and immuno-affinity-based methods (Becker-Algeri et al. 2016; Flores-Flores et al. 2018; Hashemi et al. 2016; Rastogi et al. 2004; Flores-Flores et al. 2017). Although the use of specific internal standards for quantification of individual analytes is ideal, cost and availability are an important concern, particularly in our context. For this reason, we used only two labelled mycotoxins (AFB_1-D3 and OTA-D5) as internal standards for each group. Other authors have similarly used a single internal standard to quantify mycotoxins of similar structure (Cervino et al. 2008). Overall, our findings are strengthened by the use of LC-MS/MS and stable isotope dilution, to quantify lower concentrations of mycotoxins in smaller volumes of milk.

6. CONCLUSIONS

We have developed and validated an UHPLC-MS/SRM method to quantify AFs and OTs in animal milk samples. As a preliminary study, we checked 38 raw milk samples, from a variety of animals. All samples except buffalo milk exceeded the MAL values for AFM_1 set by the EU (50 pg/mL) and four raw milk samples from cows and goats also exceeded MAL values set by

FSSAI and CODEX (500 pg/mL/0.5 µg/kg). Similar trends were seen for AFM_2 across milk samples. Further investigation with larger sample sets is warranted to understand the true extent of contamination, in addition to studies of animal food and feed from these regions of the country. Findings from this investigation suggest a need for steps to be taken to control potential fungal contamination in animal feed and to mitigate mycotoxin contamination in milk, routinely used for human consumption.

Conflicts of interest

The authors declare no conflicts of interest.

Abbreviations

Aflatoxin-AF; Ochratoxin-OT; UHPLC/MS-Ultrahigh Performance Liquid Chromatography and Mass Spectrometry; SRM-Selected Reaction Monitoring; IARC-International Agency for Research on Cancer; LOD-Lowest Detection Limit; LOQ-Limit of Quantification; LQC-Lower Quality Control; MQC-Middle Quality Control; HQC-Highest Quality Control; MAL-Maximum Allowed Level; DP-De-clustering Potential; CXP-Exit Potential; EP-Entrance Potential; CE-Collision Energy.

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Supplementary Table 1: Recovery analysis from milk and water

	% Recovery from Milk			% Recovery from Water		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
AFB1	105.0	109.2	103.6	99.1	102.4	104.0
AFB2	97.7	107.7	115.3	93.4	100.3	100.8
AFG1	101.7	108.7	116.2	98.4	101.9	100.9
AFG2	98.4	105.1	108.4	98.4	101.9	100.7
AFM1	93.2	93.7	113.4	102.1	99.5	101.2
AFM2	77.2	81.1	100.3	90.0	94.1	88.3
OTA	82.7	104.4	101.6	98.8	95.3	101.9
OTB	75.2	93.7	105.6	91.9	99.5	101.8

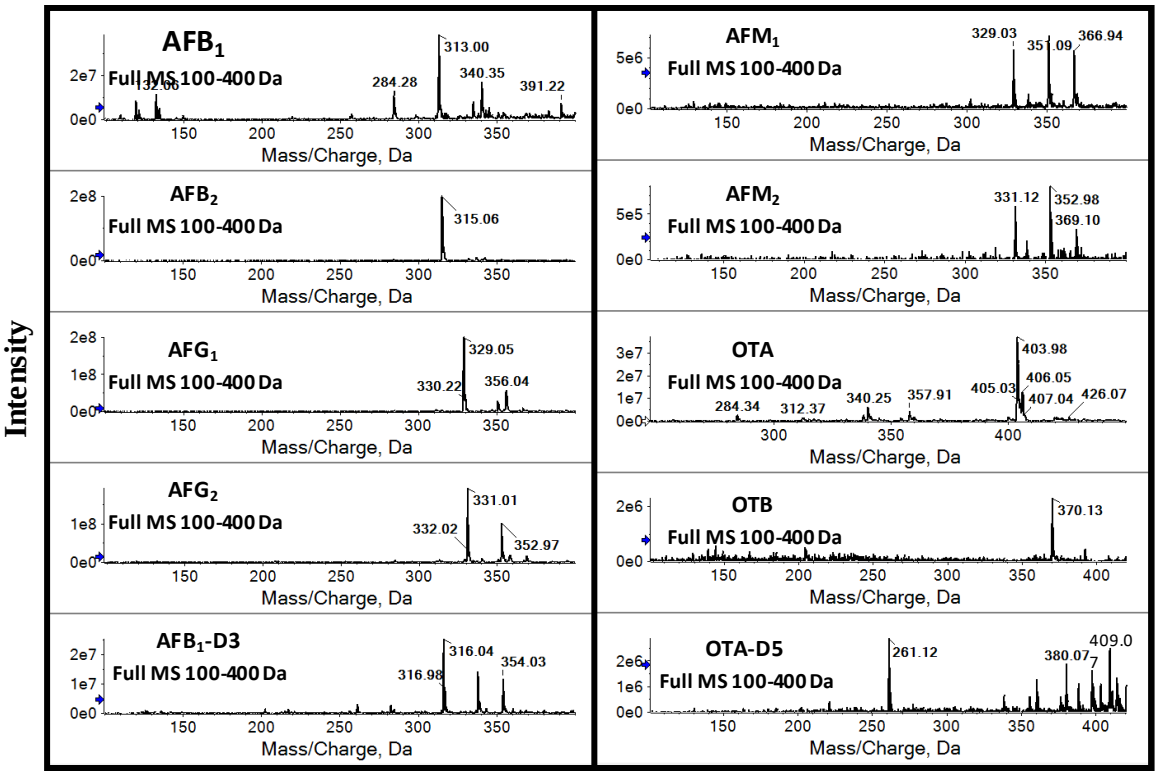
[a]LQC = lower quality control; MQC = middle quality control; HQC = high quality control

Supplementary Table 2: Effect of milk matrix on QCs

	% Recovery from milk matrix		
	LQC	**MQC**	**HQC**
AFB1	92.0	100.8	99.4
AFB2	75.4	90.9	99.2
AFG1	82.1	95.4	98.3
AFG2	92.2	98.4	100.6
AFM1	91.0	87.8	96.9
AFM2	69.0	87.5	97.5
OTA	66.7	96.1	98.6
OTB	91.5	93.9	99.1

[a]LQC = lower quality control; MQC = middle quality control; HQC = high quality control

Supplementary figure 1: Full MS spectrum of aflatoxins and ochratoxins



Chapter 4: Risk of dietary and breastmilk exposure to mycotoxins among lactating women and infants 2-4 months in Northern India

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Abstract

Mycotoxins are carcinogenic secondary metabolites of fungi that have been linked to infant growth faltering. In this study, we quantified co-occurring mycotoxins in breastmilk and food samples from Haryana, India and characterized determinants of exposure. Deterministic risk assessment was conducted for mothers and infants. We examined levels of 8 mycotoxins (Aflatoxin B_1, B_2, G_1, G_2, M_1, M_2; Ochratoxin A, B) in 100 breastmilk samples (infants 2-4 months) using ultra-high-performance liquid chromatography tandem mass spectrometry. Aflatoxin B_1, Fumonisin B_1 and Deoxynivalenol were detected in several food items (n = 298) using enzyme-linked immunosorbent assays.

We report novel data on the presence of mycotoxins in breastmilk samples from India. While breastmilk concentrations (AFM_1 median: 13.7; range: 3.9-1200 ng/L) remain low, AFM_1 was detected above regulatory limits in 27% of animal milk samples. Maternal consumption of breads ($p < 0.05$) was associated with breastmilk AFM_1 exposure.

AFB_1 (µg/kg) was detected in dried red chilies (15.7; 0-302.3), flour (3.13; 0-214.9), groundnuts (0; 0-249.1), maize (56.0; 0-836.7), pearl millet (1.85; 0-160.2), rice (0; 0-195.6), wheat (1.9; 0-196.0) and sorghum (0; 0-63.5). FB_1 (mg/kg) was detected in maize (0; 0-61.4), pearl millet (0; 0-35.4) and sorghum (0.95; 0-33.2). DON was not detected in food samples.

Our findings show the presence of Aflatoxin B_1 and M_1 at various levels of the food chain and in breastmilk, with estimated intakes exceeding provisional maximum tolerable daily intake recommendations. Further research is needed to replicate and expand on these findings in order to understand implications for maternal and child health.

Key Messages:

- AFB_1 was detected above regulatory limits in wheat, rice, millet, flour with exposure from rice and flour averaging 75.81 (range: 35.2-318.2) ng kg^{-1} bw day^{-1} and 100% of mothers exceeding provisional maximum tolerable daily intake (PMTDI) levels
- 80% of women exceeded PMTDI cut-off for AFM1 due to milk consumption
- AFM_1 was detected in 93% of animal milk (median: 125.5, range: 10.7-4158 ng/L) and 41% of breastmilk samples (median: 13.7, range: 3.9-1200 ng/L)
- While breastmilk AFM_1 concentrations remain low, 40% of samples were above PMTDI limits, 1.63(0.26-18.20) ng kg^{-1} bw day^{-1}
- Breastmilk remains the optimal nutrition source for infants and there may be elevated risks of exposure for non-exclusive breastfed children in this population due to higher levels of contamination in animal milk and stable food supply

Key words: Aflatoxins, Ochratoxins, Fumonisins, Deoxynivalenol, breastmilk, cereal crops

1. Introduction

Mycotoxins are secondary metabolites of fungi and are found in 25% of the global food system, resulting in annual losses of over 1 billion metric tons of food and feed products (Wu, Groopman & Pestka, 2014; Smith, Madec, Coton, & Hymery, 2016). In addition to being known carcinogens and genotoxins, emerging literature suggests that mycotoxins may also be associated with infant growth faltering and stunting (Lombard, 2014). Mycotoxins are found in a variety of food commodities such as maize, wheat, rice, animal source products, in addition to human milk (Bayman & Baker, 2006; Shirima et al., 2010; Sobrova, Adam, Vasatkova, Beklova, Zeman & Kizek, 2010; Wild & Gong, 2010; Smith et al., 2016; Deepa and Sreenivasa, 2017). Thus, breastmilk is a potential source of dietary exposure for infants and young children (Coppa, Khaneghah, Alvito, Assuncao, Martins, Goncalves, de Neeff, Sant'Ana, Corassin & Oliveira, 2019; Fakhri, Rahmani, Oliveira, Franco, Corassin, Saba, Rafique & Khaneghah, 2019). There are hundreds of different mycotoxins, but aflatoxins (AFs), ochratoxins (OTs), deoxynivalenol (DON) and fumonisins (FUM) are of particular consequence to public health (Warth, Braun, Ezekiel, Turner, Degen, & Marko, 2016).

Aflatoxins are produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi and AFB_1 is the most prevalent and toxic metabolite, classified as a group 1 carcinogen, known to cause cancer in humans (Shirima, Kimanya, Kinabo, Routledge, Srey, Wild, & Gong, 2013; Wild & Gong, 2010; IARC, 2006). Ochratoxins are produced by *Aspergillus ochraceus* and *Aspergillus Penicillum* (Bayman & Baker, 2006; Smith et al., 2016). OTA is classified as a group 2B agent, possibly carcinogenic to humans (Raiola, Tenore, Manyes, Meca & Ritieni, 2015). Deoxynivalenol is produced by *Fusarium graminearum* and *Fusarium culmorum* and is classified as a group 3 agent, with inadequate evidence for human carcinogenicity (Grenier &

Applegate, 2013). Fumonisins are another important group of mycotoxins produced by *Fusarium verticillioides* (*F. moniliforme*), and found predominantly as FB_1 (70%) in commodities (Deepa and Sreenivasa, 2017). Fumonisins are classified as group 2B carcinogens (Masching, Naehrer, Schwartz-Zimmermann, Sarandan, Schaumberger, Dohnal, Nagl & Schatzmayr, 2016).

Breastmilk contains elements essential for child health, growth and development (WHO, 2019). During infancy, the primary exposure source to mycotoxins is breastmilk, where lactational transfer of mycotoxins occurs via maternal diet (Warth et al., 2016). AFM_1, also known as milk aflatoxin is a hepatic hydroxylation by-product of AFB_1, is ten-fold less toxic and classified as a group 2B carcinogen (Smith, Stoltzfus & Prendergast, 2012; Etzel, 2014). AFM_1 and OTA have been detected in breastmilk samples from across the globe (Coppa et al., 2019; Fakhri et al., 2019). Infants and young children may experience the adverse effects of mycotoxin exposure up to three times greater than adults because of their larger intake/body weight ratio, higher metabolic rate and lower capacity to detoxify these contaminants (Assuncao, Vasco, Nunes, Loureiro, Martins & Alvito, 2015; Hulin, Bemrah, Nougadere, Volatier, Sirot & Leblanc, 2014; Sherif, Salama & Abdel-Wahaab, 2009).

Few studies have linked mycotoxin concentrations in breastmilk with maternal diet to understand the role of the food system as a source of exposure (Ortiz, Jacxsens, Astudillo, Ballesteros, Donoso, Huybregts & De Meulenaer, 2018). To the best of our knowledge, no studies from India and only one from the South Asia region have reported on mycotoxin concentrations in breastmilk (Khan, Ismail, Gong, Akhtar & Hussain, 2018).

Therefore, the aim of this cross-sectional observational study, was to quantify co-occurring mycotoxins in an assortment of commonly consumed cereal crops (AFB_1, FB_1 and DON), commercial infant formula (AFB_1) and 100 human milk samples (AFs B_1, B_2, G_1, G_2, M_1, M_2,

and OTs A, B) from rural and peri-urban communities in Haryana, India. These data were used to understand determinants of breastmilk mycotoxin exposure, characterize risk among mothers and children and explore variations in levels of mycotoxins across seasons (over the period of a year) and locations (peri-urban vs. rural).

2. Materials and Methods

2.1 Study subjects

The parent study was conducted in peri-urban and rural areas within Faridabad district of the northern Indian state of Haryana, with the aim to examine the role of maternal nutritional status on lactation performance. The total population of this region is 1,809,733 (2011 census) and it comprises 20 primary health centers (PHCs), with a catchment population of 30,000 – 40,000 per PHC. According to the National Family Health Survey – 4 (2015-2016), approximately 34% of children in this district are stunted, 21% are wasted and 16% of women have body mass indices of < 18.5 kg/m^2. Participants for the study were recruited from the catchments of four primary health centers (PHCs). Mothers with infants 2-4 months of age were enlisted from an existing pregnancy surveillance database, and all lactating mothers between the ages of 18-45 were eligible for enrollment. Exclusion criteria included not breastfeeding, consumption of any form of tobacco and those who were not likely to stay in the region for a period of 2 weeks post-enrollment. Written informed consent was obtained from participants. The study protocol extended over a period of 14 days and contact with the household was established to obtain information about socioeconomic status, demographic details, food insecurity, infant morbidity. A 24-hour dietary recall questionnaire was also administered.

2.2 Breastmilk sample collection

Breastmilk samples were collected at the participant's household on day one post enrollment. A cross-sectional sample of participants was enrolled over the course of twelve months (July 2017-June 2018) to capture seasonal variations. Milk samples were collected in a sterile acid washed polypropylene specimen container (Genaxy). Respondents were trained on how to manually hand-express and provide a full sample of milk from one breast. Breastmilk was gently inverted, aliquoted and stored at 2-8ºC. Samples were transported to the local laboratory in cooler boxes and maintained at -80 ºC. Stored samples were shipped to laboratories for analysis on dried ice with temperature monitoring, and maintained at -80 ºC prior to analysis. All breastmilk samples were thawed prior to processing, and each sample underwent only one freeze thaw cycle.

2.3 Food sample collection

We randomly selected ten villages stratified to represent rural and peri-urban communities from where breastmilk, animal milk and food samples were collected. Additionally, food items were sampled from seven local retail markets and three wholesale markets accessed by participants in study communities. Samples were collected at three time points, namely in May (summer), August (monsoon) and November (fall) 2018, to examine seasonal effects.

Major crops produced in the north Indian state of Haryana include wheat, barley (rabi, spring harvest), rice, sorghum, pearl millet, maize and pulses (kharif, autumn harvest) (Agri Haryana, 2018). In total, we collected 100 grams of rice, wheat, flour, chilies, maize, peanuts, pearl millet, sorghum, groundnuts, and barley samples (n = 298) and 30 commercial packaged and locally-produced buffalo milk samples. Ten commercial infant formula samples were collected in May

2019, to examine AFB_1 levels. Details of sample sizes for food items collected by season are presented in **Supplementary Table 3**.

2.4 Human and animal milk aflatoxin and ochratoxin analysis

2.4.1 Chemicals and reagents

Standards for AFB_1, AFB_2, AFG_1, AFG_2 were purchased from Sigma-Aldrich (Bangalore, India) and standards (STDs) for AFM_1, AFM_2, OTA, OTB, internal standards (ISTDs), AFB_1-D_3 and OTA-D_5 were purchased from Toronto Research Chemicals (TRC), Toronto, Canada. Purity of all standards and deuterated standards was ≥ 98%. High purity MS grade solvents (water and ACN) were procured from Honeywell, Bangalore, India. Centrifugal membrane filters (0.45 µm, PVDF) were obtained from Thermo-Fisher Scientific (Bangalore, India). Formic acid and ammonium acetate were obtained from Sigma-Aldrich (Bangalore, India). Standards were individually weighed and distributed into 1 mg/mL stocks in acetonitrile (ACN), and further diluted to 10 ug/mL aliquots in ACN and stored at -80 ºC. Working stock solutions ranged in concentration from 1 ug/mL for AFB_1, AFB_2, AFG_1, OTA, to 0.5 ug/mL for AFM_1, AFM_2 and OTB and 10 ug/mL for AFG_2.

2.4.2 Sample Preparation

Aliquoted human milk samples (1 mL) were allowed to thaw on top of ice and transferred to 15 mL centrifuge tubes. Ice-cold acetonitrile (2 mL) with 2% formic acid was then added and 10 µL of ISTDs were spiked on top. The sample was vortexed for 1 min and kept on ice for 5 min to allow for complete protein precipitation. Next, 300 µL of concentrated ammonium acetate (10 g/mL) solution was added. The sample was sonicated in a water bath sonicator for 10 min and kept on ice for 5 min, then centrifuged (5 min, 3800 x g) to separate the ACN layer.

Approximately 1.5 mL of ACN from the supernatant was transferred to 2 mL Eppendorf tube and dried in a speed vacuum. The final reconstitution was done with 100 µL of 50% ACN and filtered through a 0.45 µm PVDF membrane centrifugal filter (5 min, 1500 x g). The top 80 µL from each sample was transferred to HPLC vials and 10 µL from each was injected for analysis of mycotoxins in milk using the UHPLC-MS/SRM method. A seven-point calibration curve (ranges of 15.6-1000 ng/L for AFB_1, AFB_2, AFG_1, and OTA; 7.8-500 ng/L for AFM_1, AFM_2 and OTB and 78-5000 ng/L for AFG_2) was prepared on a daily basis, in addition to quality controls at limit of quantification (LOQ), low, medium and high-quality control (QC) levels. All animal milk samples were analyzed using UHPLC-MS/MS in a manner similar to the breastmilk samples, described above.

2.4.3 Instrumental analysis

Sample analysis was conducted using a Sciex QTRAP 6500 (Sciex Singapore) mass spectrometer, with a turbo V ion source. The mass spectrometer was coupled to an Agilent 1290 infinity II UHPLC system (Agilent Technologies India Pvt. Ltd., India), and equipped with a column oven (set to 40ºC), an auto-sampler and thermo-controller (set to 10ºC). Mobile phase solvent A was water (10 mM Ammonium Acetate, 0.1% Formic Acid) and Solvent B was acetonitrile (0.1% Formic Acid). A C-18 column (2.1 X 100 mm, 1.8 µm, Agilent, Inc) was used for separation of mycotoxins. We used an optimized gradient to achieve maximum separation (0-3 min – 10%B, 3-15 min – 10%B to 80%B, 15.1-17 min- 100%B, 17.1-22 min-10%B) at 200 µL/min flowrate. Injection volume (10 µL) was kept constant throughout these analyses. Spray voltage was set at 5500 V, curtain gas at 30 PSI, temperature at 500ºC, gas 1: 30 PSI, gas 2: 50 PSI, EP: 10 V. A scan time of 50 milliseconds per transition was used in positive ion mode. Detailed tandem mass spectrometry scans (MS/MS) were obtained using infusion of 10 µg/mL

solution of each mycotoxin in ACN and conducted in the syringe pump at a flow rate of 10 µL/min. We monitored the precursor ion and collision induced dissociation (CID) for each metabolite, which was used to generate details about product ions. We scanned quadrupole 3 to obtain product ions from m/z 50-500 with a cycle time of 1 second. We further optimized DP, CXP and CE for each intense product ion **(Supplementary Table 1)**.

2.5 Food sample AFB_1, FB_1 and DON analysis

2.5.1 Sample preparation

Food samples were analyzed using direct and indirect competitive enzyme-linked immunosorbent assays (ELISA). Approximately 100 g of each food sample was ground to a fine powder using a Kenstar Senator blender (Kenstar, Gurgaon, India). Next, 100 mL of 70% methanol (v/v-70 ml absolute methanol in 30 mL distilled water) containing 0.5% KCl was added to 20 g sample powder in an Erlenmeyer flask. For DON extraction, 100 mL deionized H_2O was used in place of methanol, in accordance to the kit manufacturer's protocol. Extracts were incubated at room temperature for 60 minutes on a revolving shaker (250 rpm), filtered through Whatman No. 4 filter paper into a fresh tube, and stored at 4ºC until ELISA analysis. A similar protocol was used to prepare a toxin free sample extract (healthy groundnut – HGN), which was used for dilution of AFB_1 and FB_1 standards and as a negative control.

2.5.2 ELISA assay procedures

For AFB_1 and FB_1, we conducted indirect competitive ELISA according to the protocols developed by ICRISAT (Reddy, Kiran Mayi, Reddy, Thirumala-Devi & Reddy, 2001). Between each step of the protocol, contents were decanted and the plate washed 3 times with a PBST wash buffer. First, ELISA plates were coated with 150 µl AFB_1-Bovine Serum Albumin (BSA; 100 ng/mL) for AFB_1 ELISAs, or FB_1-BSA (500 ng/mL) for FB_1 ELISAs, both prepared in

carbonate buffer (100 ng/mL) and incubated at 37ºC for 1 hour. Following this, blocking was conducted by adding PBST- to each well and incubating at 37ºC for 30 min. For AFB_1, we prepared standards (25-0.097 ng/mL) in 10% toxin-free extract or PBST-BSA with 7% methanol and added 100 µL to each well of the plate. For FB_1, standards were prepared similarly with concentrations ranging from 6-0.047 µg/mL. Next, we added 100 µL of diluted sample extract (1:10 in PBST-BSA), and 50 µL of antiserum diluted in PBST-BSA (1:6000 for AFB1; 1:5000 for FB_1) to all wells and incubated at 37ºC for 1 hour. Enzyme conjugation was done by adding 150 µL anti-rabbit-IgG-ALP (1:4000 in PBST-BSA) to all wells and incubated at 37ºC for 1 hour. Finally, the substrate step involved addition of pNPP prepared in 10% diethanolamine. This led to color development in 20 min, after which absorbance was read at 405 nm using a Bio-Rad iMark microplate reader (Bio-Rad Laboratories, CA, USA). The limit of detection (LOD) for the AFB_1 assay was 0.1 µg/kg and limit of quantification was 1 µg/kg, with 93% recovery (Reddy et al., 2001). LOD for FB_1 was 7.6 µg/kg and LOQ was 10 µg/kg, with recoveries ranging between 61-84% (Barna-Vetro, Szabo, Fazekas & Solti, 2000).

For DON analysis, we used a commercially-available test kit (HELICA Biosystems, CA, USA) to perform direct competitive ELISAs. The assay was conducted and performed in accordance with manufacturer's instructions. First, 200 µl of the conjugate solution was mixed with 100 µl sample extract or DON standard (10-0 ug/mL) in a 96-well dilution plate. After dilution, 100 µl of the contents from each dilution well were transferred to the corresponding Antibody Coated Microtiter Well of the kit's test plate and incubated at room temperature for 15 minutes. The contents of the test plate were discarded, and the plate was washed 3 times with a PBST wash buffer. We then added 100 µl of the Substrate Reagent to each well and incubated at room temperature for 5 minutes. Finally, 11 µl of Stop Solution was added to the plate in the

same sequence as the Substrate Reagent. Absorbance was read at 450 nm using the same instrument as described above. The LOQ for DON was 10 µg/kg (HELICA Biosystems, CA, USA).

2.5.3 ELISA data analysis

All samples were assayed in duplicate on the ELISA plates. Optical Densities (OD) were recorded and processed using Microplate Manager 6 software (Bio-Rad Laboratories, CA, USA). For AFB_1 and FB_1, second-order polynomial standard curves were generated for each plate, plotting Log_{10} values of standard concentrations on the y-axis and OD values on the x-axis. For DON, standard curves were generated according to manufacturer's instructions by calculating % bound (%B/Bo) for each standard and plotting Logit %B/Bo on the y-axis against Log_{10} DON concentration on the x-axis. For all toxins, the standard curves were used to compute sample concentrations by interpolation, taking all sample dilution factors into account. Samples with OD values outside the OD range of the standards were serially diluted and re-analyzed until their OD values were within range.

2.6 Dietary intake data for mothers and infants

A food frequency questionnaire (FFQ) and multiple pass 24-hour dietary recall was collected from each respondent during a household visit. A semi-quantitative FFQ validated as part of the Indian Migration Study (IMS) was implemented and items were divided into groups namely, *breads and rotis*; *grains and staples*; *dairy, eggs and animal fat*; *fruits and vegetables*; *lentils*; *meats and sweets and snacks* (Bowen, Bharathi, Kinra, DeStavola, Ness & Ebrahim, 2012). The 24-hour recall data were entered into DietCal, a software developed by the All India Institute of Medical Sciences (AIIMS, 2015).

Infant breastmilk intake was assessed using deuterium oxide dose-to-mother technique, where a single dose of 30 grams of 99.8% deuterium oxide (D_2O) was administered to lactating mothers on day 0 of enrolment (IAEA, 2010). Saliva collection procedures were based on methods prescribed by the IAEA human health series (Number 7) (IAEA, 2010). Samples were stored in 2 mL acid washed cryogenic vials at -20ºC. Analysis of saliva samples was conducted using Fourier Transform Infrared Spectrometry (FTIR), which provided the exact volume of breastmilk consumed by each child (L/day). Infant body weight was measured using a SECA 385 (SECA gmbh & Co., Hamburg, Germany; accuracy 20 gm) and length was measured using a SECA 417 (SECA gmbh & Co., accuracy 1mm). All measures were taken in duplicate.

2.7 Statistical analysis of data

All statistical analyses were conducted in SAS 9.4 (SAS Institute, Cary, NC). Maternal and demographic characteristics are expressed as percentages and means ± SD. A household wealth index was derived using principal components analysis (PCA) and the Household Food Insecurity and Access Scale (HFIAS) was used to categorize households by food security status (FANTA, 2007).

Breastmilk and food sample mycotoxin results are presented as medians and interquartile ranges (IQR). Left censored data were handled by replacing the values for each mycotoxin, below the limit of detection with the medium bound value or ½ that of LOD (EFSA, 2010). Outlier values (± 3 SD) were removed and non-normally distributed data were log transformed.

TOBIT (PROC QLIM) or censored regression models were generated to identify factors significantly associated with breastmilk mycotoxin concentrations using a two-step approach. Variables with a $p < 0.1$ in bivariate analyses were included in multivariable models by category of determinants (sociodemographic, dietary, environmental), controlling for maternal age and

wealth status. A p-value < 0.05 was considered statistically significant. For analysis of dietary determinants, we used frequencies for consumed vs. not consumed.

ANOVAs and Kruskal-Wallis tests were used to assess differences in mean concentrations of AFB_1 and FB_1 in food items by season and location of collection (rural/village level markets, peri-urban/retail markets and urban/wholesale markets).

2.8 Dietary exposure assessment and risk characterization

Estimated daily intake (EDI) of AFB_1 and AFM_1 for mothers via consumption of a variety of food items and animal milk and of AFM_1 for infants through breastmilk were calculated using a deterministic exposure assessment approach (Assuncao, Vasco, Nunes, Loureiro, Martins, & Alvito, 2015; Cantu-Cornelio, Aguilar-Toala, Rodriguez, Esparza-Romero, Vallejo-Cordoba, Gonzale-Cordova, Garcia, Hernandez-Mendoza; 2016; Ortiz et al., 2018). EDIs were calculated using the equation below and reported as ng kg^{-1} bw day^{-1} for relevant mycotoxins in each food item for which consumption data were available (Ortiz et al., 2018).

Estimated daily intake = *average concentration of mycotoxin (ng/kg or ng/L) x consumption of item (kg/day or L/day) ÷ body weight (kg)*

Average concentrations of mycotoxins in food items and milk were derived from study samples. Average consumption of food items (in kg/d or L/day) was derived from 24-hour recall data for mothers and deuterium oxide (D_2O) results for infant breastmilk volume consumed. Daily intake values were compared to reference dose values as part of risk characterization. As aflatoxins are carcinogenic compounds, exposure at any level is considered unsafe and levels of such substances should be as low as reasonably achievable (ALARA) (JECFA/EFSA, 2007). A provisional maximum tolerable daily intake (PMTDI) value of 1 ng kg^{-1} • bw day^{-1} for aflatoxins has been proposed for children and adults without hepatitis-B (Kuiper-Goodman, 1995, 1998).

The extent to which exposure exceeds these values was used to assess risk (Cunha, Sa, & Fernandes, 2018).

The European Food Safety Agency (EFSA) has also proposed the use of margin of exposure (MOE) for risk analysis of aflatoxins. MOE represents the ratio between BMDL10 (benchmark dose lower confidence limit for 10% extra risk of liver tumor formation in rats amounting to 170 ng kg^{-1} bw day^{-1}) and EDI (EFSA, 2005). Here, an EDI is considered of concern to public health if the MOE is lower than 10,000. MOE values do not quantify risk, but are used to indicate a level of concern. A lower MOE indicates a higher level of concern (EFSA, 2005).

3. Results

3.1 Analytical validation

The occurrence of 6 aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2) and 2 ochratoxins (OTA, OTB) were investigated in human and animal milk samples. Method performance for the assay was in accordance with guidelines set by the USFDA (Guidelines for Bioanalytical Method Validation, 2018) and results are presented in **Supplementary Table 2**. All analytes presented a good linear response (R^2 between 0.9991-0.9999) and recoveries were > 75% for standards spiked on top of milk and > 88% for standards spiked to water.

3.2 Population Characteristics & Occurrence of Aflatoxins and Ochratoxins in Human Milk

Sociodemographic characteristics of households, in addition to details about mothers and children in our sample are presented in **Table 1**.

Table 1: Household, maternal and child characteristics of N = 100 participants in Haryana, India

Household Characteristics (N)	**%**
Religion	
Hindu	71
Muslim	27
Other	2
Caste	
Scheduled caste	26
Other backward caste (OBC)	50
Other caste	24
Paternal Occupation	
Not working/retired/unemployed	5
Cultivator (own land)	2
Business/petty trader/self-employed	20
Salaried employee	49
Other	24
Socio-economic Status	
Low	33
Middle	34
High	33
Residence	
Peri-urban	77
Rural	23
Food Insecurity	
Food secure	82
Food insecure	18
Maternal Characteristics	**Mean (±SD)**
Maternal Age (years)	24.9 (3.6)
Maternal Education (years)	6.8 (5.1)
Parity	2.3 (1.3)
Anthropometry	
Weight, kg	52.0 (9.97)
Height, cm	152.7 (6.0)
BMI, kg/m^2	22.0 (3.7)
BMI categories, kg/m^2	
Underweight (< 18.5)	11
Normal (18.5-25)	69
Overweight (25-30)	17
Obese (> 30)	3
Current Dietary Patterns	
Vegetarian	36
Non-vegetarian	53
Eggetarian	11

Child Characteristics	%; Mean (±SD)
Child Sex	
% Male	51
% Female	49
Child Age (months)	3.07 (1.99, 4.05)
Child Nutritional Status	
% Stunted (length-for-age z-score < -2)	18
% Underweight (weight-for-age z-score < -2)	16
% Wasted (weight-for-length z-score < -2)	8
Current breastfeeding practices	
Currently exclusively breastfeeding	28

AFM_1 was detected in 41 human milk samples. Contamination levels in milk ranged between 0-1200 ng/L for AFM_1. Only one sample was detected with AFM_1 levels above Food Safety & Standards Authority of India (FSSAI) set limits in animal milk (500 ng/L) **(Table 2)**. There are currently no cut-offs for aflatoxins in human milk samples as these are not commercially regulated.

Table 2: Concentrations (ng/L) of Aflatoxins and Ochratoxins in human breastmilk (n = 100)

Mycotoxin	LOD	LOQ	% samples > LOD[a]	Median[b]	Inter-quartile range (IQR)	Min[c]	Max	N > regulatory limits	Maximum regulatory limits[d]
	(ng/L)			(ng/L)					(ng/L)
Aflatoxin B_1	15.6	31.3	8	22.9	7.4	7.8	27.1		100
Aflatoxin B_2	15.6	31.3	43	43.0	49.0	7.8	265.0		-
Aflatoxin G_1	15.6	31.3	5	23.7	7.3	7.8	45.3		-
Aflatoxin G_2	156	313	19	121.0	71.7	39.0	241.0		-
Aflatoxin M_1	7.8	15.6	41	13.7	16.3	3.9	1200.0[e]	1	25 500[f]
Aflatoxin M_2	7.8	15.6	18	15.0	9.1	3.9	33.9		-
Ochratoxin A	15.6	31.3	13	20.0	13.2	7.8	39.8	-	500
Ochratoxin B	7.8	15.6	42	11.9	23.8	3.9	62.2		-

[a]Number of samples below the limit of detection and assigned a value of 0.5 LOD
[b]Median of values > LOD
[c]Values corresponding to 0.5 LOD for each mycotoxin (Ortiz et al., 2018)
[d]Maximum regulatory limits set for infant formula, follow-on formula, baby foods for infants and young children by the European Union
[e]Outlier excluded from further analysis
[f]Maximum regulatory limits set for animal milk by FSSAI

TOBIT regression analyses of the determinants of AFM_1 exposure in human milk samples showed maternal consumption of breads to be significantly ($p < 0.05$) associated with an increase in breastmilk concentrations of AFM_1. This trend remained significant for bread consumption after adjusting for maternal age and household wealth index (**Table 3**). Non-significant positive trends were seen for associations between consumption of curds, snacks and sweets and AFM_1 concentrations.

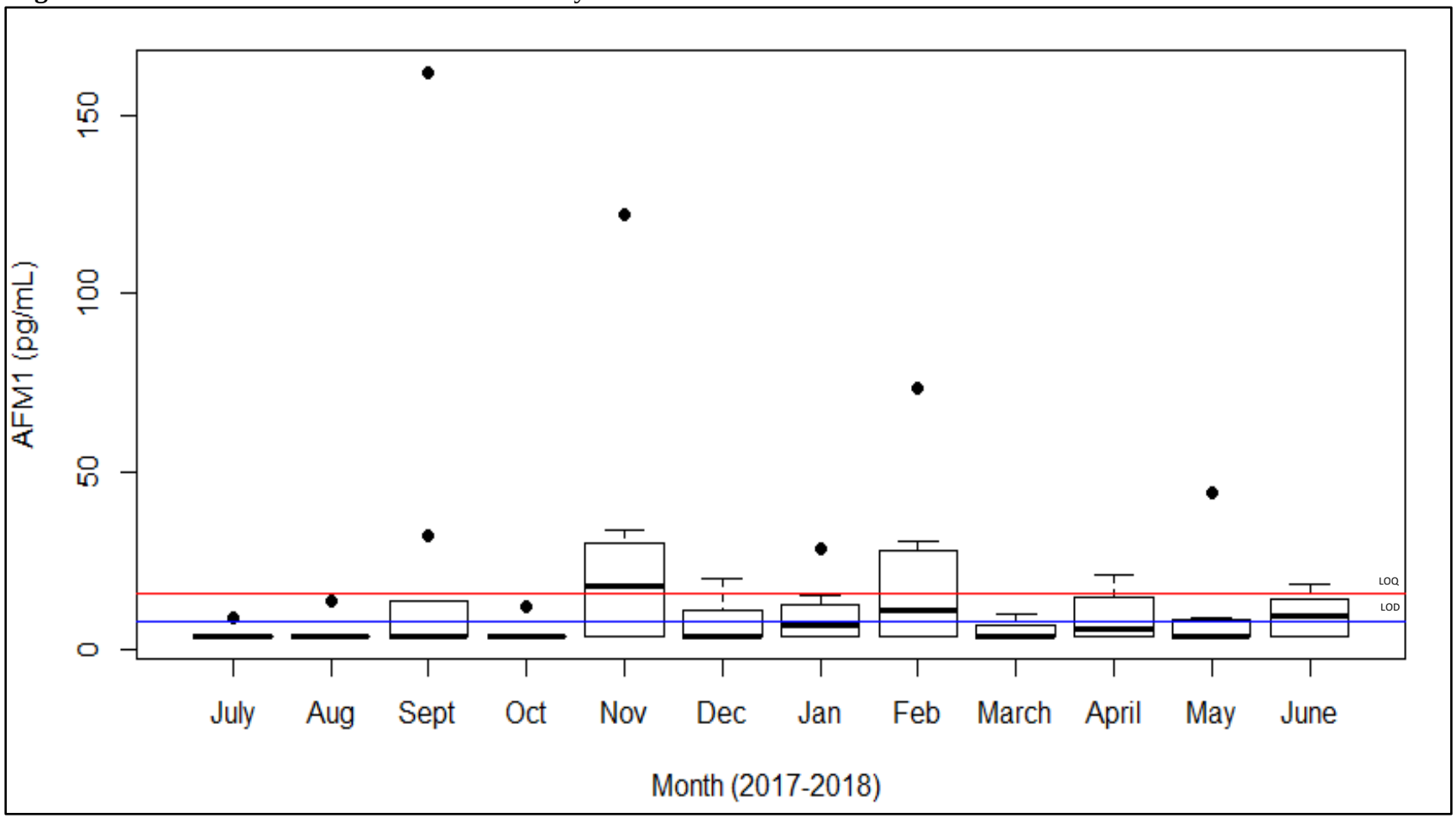
Table 3: Determinants of AFM_1 exposure in breastmilk samples

	Univariate TOBIT Regression		**Multivariable TOBIT Regression**	
	AFM_1 β (95% CI)[b]	**p-value**	**AFM_1 β (95% CI)[b]**	**p-value[c]**
Maternal Age	0.94 (0.15, 7.51)	0.27	0.92 (0.15, 7.51)	0.16
Wealth Index				
Low	0.98 (0.23, 11.76)	0.97	1.20 (0.23, 11.67)	0.71
Middle	1.10 (0.23, 11.71)	0.84	1.32 (0.23, 11.61)	0.58
High	*REF*		*REF*	
Dietary Variables				
Breads	0.36 (0.22, 10.93)	**0.02**	0.32 (0.22, 10.99)	**0.01**
Curd	0.44 (0.23, 11.37)	0.08	0.52 (0.22, 11.28)	0.16
Snacks & Sweets	0.28 (0.28, 14.15)	0.07	0.35 (0.28, 13.95)	0.12

[a]Multi-variable models adjusted for maternal age and wealth index
[b]Log transformed values back-transformed (presented in table) for interpretation of β coefficient
[c]No significant associations with sociodemographic and environmental variables

No statistically significant trends were observed for sociodemographic variables (maternal age, parity, years of schooling), maternal biological characteristics (weight, body fat, body mass index, fat mass index), household characteristics (religion, caste, HH wealth index, food security index, region of residence (peri-urban/rural), paternal occupation) and/or environmental variables (location of procurement of staple crops, self-reported instances of insect infestation and moisture in household storage of crops). We did not see statistically significant seasonal differences in values of AFM_1 in human milk samples across the 12-month span for which data were collected **(Figure 1)**.

Figure 1: Breastmilk Aflatoxin M_1 concentrations by month[a,b]



[a]N per month, Monsoon: - July: 7, Aug: 8, Sept: 9, Fall: - Oct: 8, Nov: 9, Winter: - Dec: 9, Jan: 8, Spring: - Feb: 9, March: 8, Summer: - April: 8, May: 9, June: 8
[b]N for AFM_1: 99, after removing outlier ([AFM_1] of 1200 pg/mL

Mean concentration of OTA in our study was 23.15 (± 7.78) ng/L, and only two samples were above the limit of quantification. Other mycotoxins were detected at levels < LOQ in our study, and have therefore not been considered for further statistical analyses. Median (IQR) concentrations are presented in **Table 2.**

3.2.1 Occurrence of Aflatoxins and Ochratoxins in Animal Milk

AFM_1 was detected across all 30 samples of animal milk **(Supplementary figure 2)** at levels above the 7.8 ng/L, limit of detection. Median (IQR) values by season were as follows: - summer: 102.45 (178.80); monsoon: 50.35 (165.70); fall: 389.21 (1646.33). Eight (27%) animal milk samples were over the 500 ng/L regulatory limit set by the Food Safety and Standards Authority of India (FSSAI). Other mycotoxins including AFB_1 (n = 19), AFB_2 (n = 23), AFG_1 (n = 1), AFM_2 (n = 23) were seen in several samples at levels above the limit of detection **(Supplementary figure 2)**. OTA and OTB were not detected in any of the animal milk samples in this study.

3.3 Occurrence of Aflatoxin B_1, Fumonisin B_1 and Deoxynivalenol in Food Samples

Aflatoxin B_1 was detected across a range of food items at all three seasonal timepoints and several food samples were above FSSAI set regulatory limits. Median sample AFB_1 concentrations by food items are presented in **Supplementary table 3**. We saw statistically significant ($p < 0.05$) variations in AFB_1 levels across seasons for wheat, sorghum, flour, groundnuts, rice and pearl millet, with highest mean concentrations observed during the monsoon collection period **(Supplementary table 3)**. Negligible levels of AFB_1 were detected in 10 commercial infant formula samples collected in our study.

Fumonisin B_1 was analyzed only in maize, pearl millet and sorghum, given the known vulnerability of these commodities to infestation by implicated fungi. The toxin was detected in

these commodities across the three seasonal time points **(Supplementary table 3)**. Samples were above regulatory limits set by the European Union for maize (0.2 mg/kg) at all three time points. We saw statistically significant seasonal trends for mean concentrations of FB_1 in pearl millet and sorghum samples. Both pearl millet and sorghum are harvested in autumn, which likely explains non-detected levels of FB_1 contamination during the fall collection period. We did not see any statistically significant trends for variation in mean concentrations of AFB_1 or FB_1 among rural and peri-urban sites and between types of markets, namely village, mid-retail and wholesale levels. Deoxynivalenol was not detected in any of the food samples in our study, likely owing to low disease pressure by *F.graminearum* and the generally warm climate in the study region.

3.4 Deterministic Exposure Assessment and Risk Assessment

Maternal exposure assessment to mycotoxins through intake of animal milk, flour and rice was conducted. Data from the 24-hour recall show that 93% of women in our sample consumed milk at an average volume of 0.33 L/day (± 0.30), 92% consumed wheat flour at 0.24 kg/day (± 0.10) and rice was consumed by 21% of our sample at 0.19 kg/day (± 0.14). Mean maternal estimated daily intake of AFM_1 due to milk was 3.58 (range: 0.42-17.74) ng kg^{-1} bw day^{-1}. Mean estimated daily intake for AFB_1 via flour was 82.3 ng kg^{-1} bw day^{-1} and 46.4 ng kg^{-1} bw day^{-1} via rice **(Table 4)**.

Table 4: Estimated daily intake (EDI) using a deterministic approach for mothers and infants[a]

Commodity		Mean Intake	SD	P50	P75	P90	P95	P97.5	P99
		(kg/day)							
Flour	Mother	0.24	0.10	0.24	0.30	0.36	0.40	0.47	0.49
Rice	Mother	0.19	0.14	0.15	0.20	0.40	0.50	0.50	0.50
Animal milk	Mother	0.33	0.30	0.25	0.45	0.66	0.98	1.02	1.18
Breastmilk	Infant	0.75	0.17	0.73	0.86	0.96	1.01	1.19	1.25
Commodity		**Mean Estimated Daily Intake AFB_1/AFM_1**	**SD**	**P50**	**P75**	**P90**	**P95**	**P97.5**	**P99**
		(ng/kg • bw/day)							
Flour	Mother	82.3	36.9	80.55	106.7	131.3	149.2	162.5	172.0
Rice	Mother	46.4	35.8	36.7	67.1	98.2	121.9	125.1	127.0
Animal milk	Mother	3.58	3.20	2.58	5.09	7.40	9.62	11.55	13.03
Breastmilk	Infant	1.63	2.78	0.67	1.70	3.19	4.61	14.03	18.20

[a]N = 99, values for EDI calculations do not include AFM_1 outlier for child intake

Using a deterministic risk assessment approach, 80% of women exceeded this cut-off for AFM_1 due to milk consumption; 100% of our sample significantly exceeded the provisional maximum tolerable daily intake level of 1 ng kg^{-1} bw day^{-1} for AFB_1 due to consumption of rice and wheat flour (Kuiper-Goodman, 1998; Yogendrarajah et al., 2014). MOE values for maternal aflatoxin exposure from flour (range: 0.90 – 17.21), rice (range: 1.33 – 17.54) and milk (range: 9.58 – 404.53) were also lower than 10,000 indicating that exposure to AFB_1 and AFM_1 in the food system are of priority for risk management actions (Ortiz et al., 2018).

Mean estimated daily intake value of AFM_1 from breastmilk for infants in our study was 1.63 ng kg^{-1} bw day^{-1}, 40% of the sample exceeded PMTDI levels with upper percentiles of milk consumption exceeding thresholds by 3-18 folds (P90-P99). Approximately 16% of infants in our sample consumed animal milk, 7% consumed formula, 5% received food grain and/or porridge made of grain, in addition to breastmilk. Thus, exposure to mycotoxins in dairy milk and via contaminated complementary foods may be of concern among children in this population.

4. Discussion

We found AFM_1 in 41 out of 100 breastmilk samples analyzed for mycotoxins in India at concentrations ranging between 3.9-1200 ng/L. Studies from countries including Iran, Brazil, Turkey, Italy, Egypt, Jordan, Germany, Nigeria among others, have quantified AFM_1 in human milk samples at concentrations ranging between 0-19,000 ng/L (Coppa et al., 2019). Fakhri et al., (2019) in a recent systematic review, noted an overall pooled concentration of AFM_1 across 196 global studies to be 27.67 (95% CI: 26.67-28.67 ng/L) (Fakhri, Rahmani, Oliveira, Franco, Corassin, Saba, Rafique & Khaneghah, 2019). Samples from our study show concentrations to

be within the ranges described by others, although no other data on breastmilk mycotoxins from India currently exists in the literature. The overall prevalence of AFM_1 in breastmilk from studies conducted globally, is highly variable and associated with mycotoxin contamination in food items and in turn, maternal dietary patterns, which differ across countries (Cherkhani-Hassani et al., 2016).

Our findings suggest that consumption of bread among women in this population is associated with increased concentrations of AFM_1 in milk. This is likely due to the presence of AFB_1 in flour used to produce bread. Carry-over of AFB_1 from diet and excretion as AFM_1 in breastmilk is estimated to be between 0.1 and 0.4% (Zarba, Wild, Hall, Montesano, Hudson & Groopman., 1992). Prior studies have found maternal consumption of cereals, peanut butter, vegetable oil, rice (Elzupir, Abas, Fadul, Modwi, Ali, Jadian et al., 2012), cow milk (Mahdavi, Nikniaz, Arefhosseini, & Jabbari, 2010) and sausage (Jafarian-Dehkordi & Pourradi, 2013) to be associated with increased concentrations of AFM_1 in breastmilk from women in Iran. A study conducted in Italy found that lactating women with high AFM_1 (140 ng/L) in breastmilk had consumed a large amount of corn meal-based foods in substitution for cereal-based food such as rice, pasta, bakery products and breakfast cereals (Galvano et al., 2008). Consumption of corn oil, peanuts, raw milk, beans and wheat meal have been associated with higher breastmilk AFM_1 in Egypt and Nigeria (Polychronaki, Turner, Mykkanen, Gong, Amra, Abdel-Wahhab & El-Nezami, 2006; El Tras et al., 2011; Adejumo et al., 2013). Higher consumption of rice and chocolate have also been associated with AFM_1 in milk (Bogalho et al., 2018).

Although we did not see any significant trends, previous studies have shown associations between AFM_1 concentrations in breastmilk and season of sample collection, lower educational levels and stage of lactation (Adejumo et al., 2013; Bogalho et al., 2018; Polychronaki et al.,

2006). Bogalho et al., (2018) noted that breastmilk collected at early stages of lactation featured mean concentrations of AFM_1 higher than samples collected 6-12 months postpartum.

Several additional determinants of lactational transfer of mycotoxins, have been identified, including but not limited to maternal dietary diversity and hydration, frequency of infant feeding and breast infection (Warth et al., 2016). There remains a dearth of knowledge about the lactational transfer rates of mycotoxins from blood to breastmilk. Milk to plasma ratios for AFM_1 were estimated to be 0.21 among nursing Egyptian mothers (Hassan, Sheashaa, Fatah, Ibrahim & Gaber, 2006), although this metabolite is transient in plasma, and concentrations of AFM_1 in breastmilk are higher at earlier stages of lactation (Degen et al., 2013; Polychronaki et al., 2006). We did not detect OTA in breastmilk samples in our study. Only a few studies from countries including Brazil, Iran, Chile, Italy and Turkey have documented OTA in breastmilk at ranges between 0-13,111 ng/L (Coppa et al., 2019).

Estimated daily intake values for AFM_1 in breastmilk in our study ranged between 0.26 – 18.20 ng kg^{-1} bw day^{-1}, whereas others conducted globally have documented ranges between 0.003-917 ng kg^{-1} bw day^{-1}, accounting for variations in prevalence and concentrations of mycotoxins across regions (Coppa et al., 2019).

Our findings for AFM_1 in animal milk samples are in concordance with a large nationally representative survey conducted by the FSSAI in 2018, which found 5.7% ($n > 6000$) of milk samples in India to be contaminated with AFM_1 (FSSAI, 2019). Other studies conducted in India, have shown AFM_1 contamination at ranges of 65-1012 ng/L in milk products, 28-164 ng/L in liquid milk; and in animal milk samples from southern India at ranges between 100-3800 µg/L (Rastogi, Dwivedi, Khanna & Das, 2004; Siddappa, Nanjegowda & Viswanath, 2012).

Our findings for AFB_1 in food items are in alignment with others that have documented levels of aflatoxins in maize, groundnuts, spices, peanuts, corn, rice, soybeans, sorghum, cereals, and chilis at levels ranging between 0-46,000 µg/kg (Reddy, Salleh, Saad, Abbas, Abel & Shier, 2010). Higher levels of AFB_1 in food samples collected during the monsoon season in our study are likely due to increased moisture levels and humidity, known to impact the growth of mycotoxigenic mold (Ojuri, Ezekiel, Eskola, Sarkanj, Babalola, Sulyok, Hajslova, Elliott, Krska, 2019). A large multicenter study conducted in India, found 40.3% of wheat samples collected from across the country to have AFB_1 levels $\geq$ 5 µg/kg, with 16% of samples over the Indian permissible regulatory limit of 30 µg/kg, when that study was published (current legal limit in India is 15 µg/kg) (Toteja, Mukherjee, Diwakar, Singh et al., 2006). Additional studies of items intended for animal feed from India have found aflatoxin contamination in groundnut cake, maize, millets, rice bran, sorghum, soybeans, sunflower and mixed feeds in excess of 10 µg/kg (Thirumala-Devi, Mayo, Reddy, & Reddy, 2002).

Cumulative estimated daily intake of AFB_1 due to consumption of rice and flour ranged between 35.2-318.2 ng kg^{-1} bw day^{-1}. Dietary aflatoxin B_1 exposure between 20 – 120 µg kg^{-1} bw day^{-1} for periods of 1-3 weeks or consumption of staple foods containing concentrations of AFs of 1 mg/kg or higher are suspected to cause acute aflatoxicosis and possibly death (JECFA, 2016). Overall levels of AFB_1 exposure in our study are significantly below these thresholds.

Our findings for Fumonisin B_1 in maize, sorghum and millet are in accordance with other studies that have documented FB_1 and AFB_1 in these items, in addition to poultry feed from India (Shetty & Bhat, 1997). We did not detect DON in our study, however, other studies conducted in northern India have detected the mycotoxin in upward of 30% of cereal crop samples collected, with 7% exceeding FSSAI set limits of 1 mg/kg (ranges: 0.01 – 4.73 mg/kg) (Mishra, Ansari,

Dwivedi, Pandey, & Das, 2013). More recently, a study conducted in India found 51.7% of food samples commonly consumed by infants and young children to be contaminated with DON at levels significantly above the JECFA prescribed provisional maximum tolerable daily intake (PMTDI) level of 1 µg/kg-bw (JECFA, 2010; Gummadiadala, Chen, Miller, Mitra, Banaszek et al., 2016).

Additional sources of dietary exposure to AFs and other mycotoxins not examined in this study are likely in this population, and require further investigation, in conjunction with the additive and synergistic effects of such exposures on health (Smith, Madec, Coton, & Hymery, 2016). Overall maternal EDIs for AFB_1 and AFM_1 are higher than those in infants. This suggests that the toxicity of mycotoxins is reduced as they move down the food chain. Breastmilk, although a potential source of toxin exposure is significantly less harmful to infants as compared to other dietary sources that may be contaminated.

The availability of mycotoxin biomarker data in serum samples would also be an important asset to a study of this nature. Dietary mycotoxins are absorbed from the small intestine and transferred to serum, from where they would be excreted into breastmilk during lactation. It is likely that higher concentrations of dietary mycotoxins will be detected in serum to represent the true extent of exposure.

Although limited by a small sample size, we have used gold standard methods for detection and quantification of mycotoxins in human milk samples. We were also able to assess seasonality in relation to concentrations of mycotoxins at various levels of the food ecosystem and from breastmilk within study communities. High quality data were collected for our 24-hour recalls using validated instruments from the Indian Migration Study and portion sizes and

amount consumed were estimated using calibrated measuring equipment commonly found in Indian kitchens (Bowen et al., 2012).

We were not able to calculate estimated daily intake values for all foods consumed by mothers in our sample. It is therefore likely that cumulative exposure is higher than noted here, from other sources, in addition to exposure to other mycotoxins not quantified in our study. It is also critical to investigate mycotoxin exposure among infants who are not being exclusively breastfed, and those consuming weaning foods, as exposures are likely to be higher in these groups. Studies in East Africa have shown exposure to AFs and FBs in infant foods to be of concern among non-exclusively breastfed children under 6 months of age (Magoha, Kimanya, De Meulenaer, Roberfroid, Lachat & Kolsteren, 2016).

Due to constraints associated with maintaining our cold chain and ensuring integrity of biological samples during collection, from field to lab, we were limited in the locations from where breastmilk samples were collected. To this end, the overall generalizability of our findings to communities across Faridabad district and the state of Haryana more broadly, is of concern. Future studies should consider more representative sampling of study sites from peri-urban and remote and rural communities in the state. Exposure to mycotoxins has been linked to poverty, it is therefore advisable to sample from communities of the lowest socioeconomic denominations to ensure true representation (LeRoy et al., 2015)

Finally, there is a need for longitudinal cohort studies, complemented with total diets data to examine exposure to mycotoxins across the different stages of lactation, particularly in light of mixed evidence regarding their effects on infant growth faltering and stunting (LeRoy, Sununtnasuk, Garcia-Guerra & Wang, 2017; Hoffman, Jones & LeRoy, 2018; Passarelli, Bromage, Darling, Wang, Aboud, Mugusi, Griffiths & Fawzi, 2019).

5. Conclusion

Breastmilk remains the best source of essential nutrients and immune factors for infants and exclusive breastfeeding is the recommended standard of practice for children under 6 months (WHO, 2019). Although AFM_1 was detected in 41% of breastmilk samples, concentrations of the aflatoxin remain low and below FSSAI set regulatory limits. Exposure assessments suggest levels of aflatoxins in food are higher than permissible limits. Women who consume flour-based products such as bread are at increased risk of mycotoxin exposure, in this population. Between 80-100% of women in our sample had dietary intakes of AFs above recommended daily limits of exposure. There remains a need for further investigation to understand the extent of contamination across the food chain, from feed to child.

6. References

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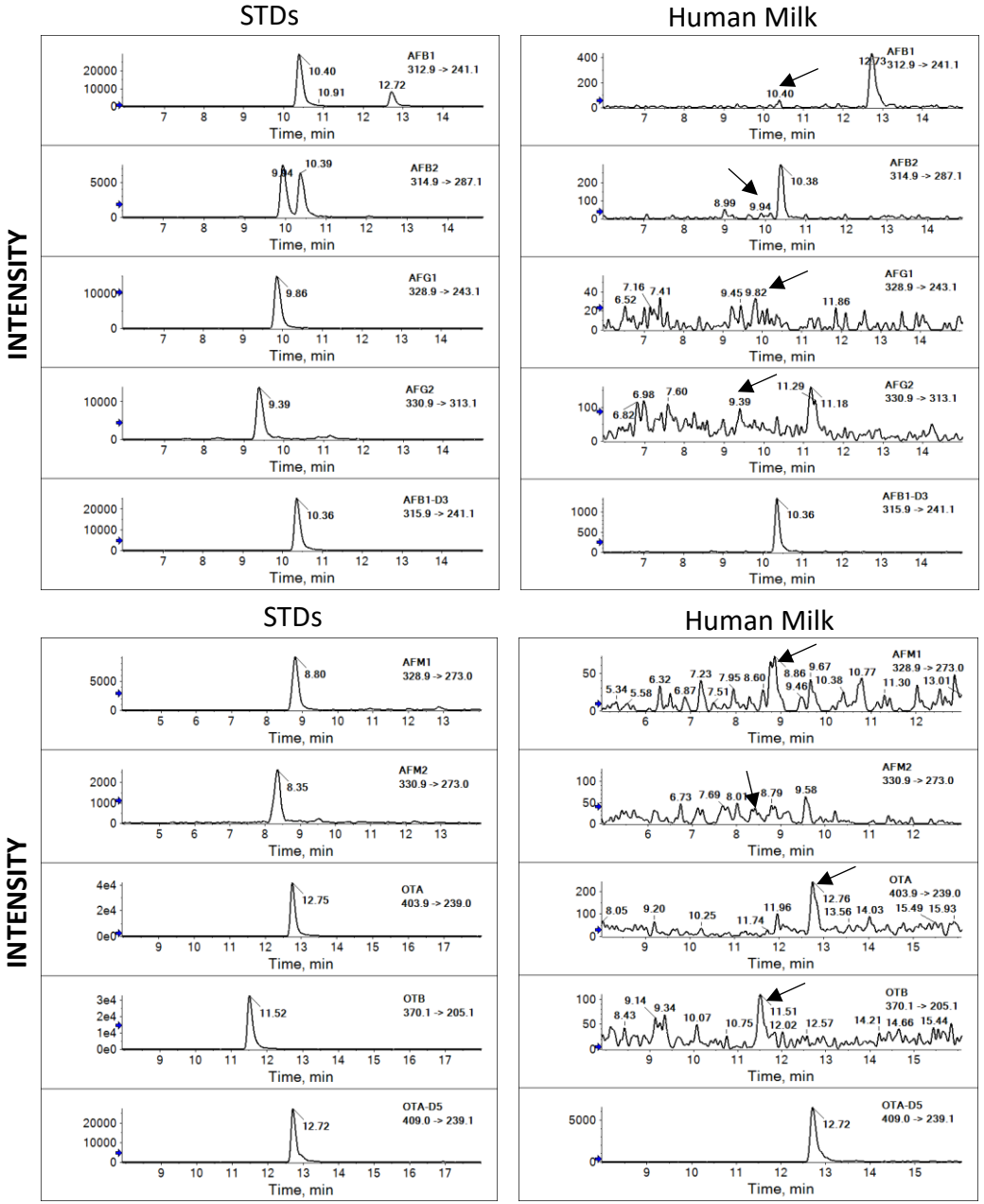
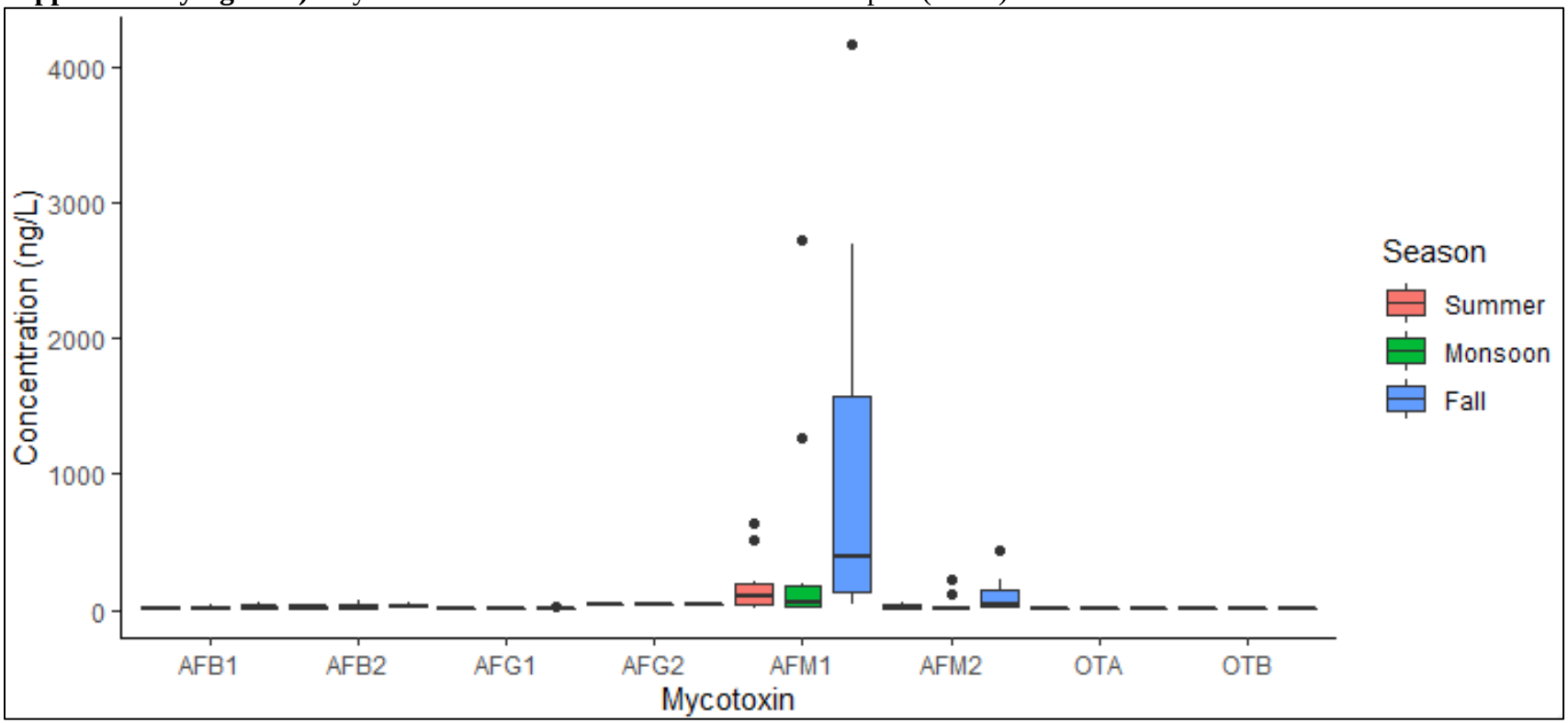
891-894. Doi: 10.1093/carcin/13.5.891.

Supplementary figures 1 a-d): Chromatograms for AFs & OTs in standards & human breastmilk



Supplementary figure 2): Mycotoxin concentrations in animal milk samples (N=30)[a,b,c]



[a]Values for milk samples < LOD were replaced with 0.5 LOD value for each metabolite
[b]Fresh buffalo and commercial packaged cow milk samples tested
[c]No significant differences in AFM_1, AFM_2 levels by season

Supplementary Table 1: Selected Reaction Monitoring Table

Analyte	Parent ion (m/z)	Product ion (m/z)	Retention time (min)	De-clustering Potential (volts)	Collision Energy (CE)	CXP (volts)
AFB_1	312.93	241.08	10.4	161	53	30
AFB_2	314.95	287.08	9.9	176	39	26
AFG_1	328.92	243.05	9.9	161	39	22
AFG_2	330.96	313.13	9.4	131	35	32
AFM_1	328.96	273.04	8.8	171	33	38
AFM_2	330.92	273.05	8.4	146	33	32
AFB_1-D_3	315.95	241..09	10.3	186	33	28
OTA	403.94	239.02	12.7	61	21	20
OTB	370.06	205.06	11.5	76	27	22
OTA-D_3	409.02	239.05	12.7	16	33	32

Supplementary Table 2: Performance characteristics of LC-MS/MS analytical method

Analyte	Calibration range	R^2	LOD	LOQ	Apparent Recovery	CV
	(pg/mL)		(pg/mL)		(%)	(%)
AFB_1	15.6-1000	0.9995	15.6	31.3	99.3	5.91
AFB_2	15.6-1000	0.9995	15.6	31.3	96.0	3.73
AFG_1	15.6-1000	0.9999	15.6	31.3	100.75	2.76
AFG_2	78-5000	0.9998	78	156	101.67	0.67
AFM_1	7.8-500	0.9998	7.8	15.6	99.49	3.80
AFM_2	7.8-500	0.9999	7.8	15.6	98.72	3.90
OTA	15.6-1000	0.9963	15.6	31.3	100.51	3.87
OTB	7.8-500	0.9935	7.8	31.3	98.46	5.88

%CV = coefficient of variation

Supplementary Table 3: Concentrations of AFB_1 and FB_1 in food items by season

Food Item	Mycotoxin	Regulatory Limit (µg/kg)[a]	Summer			Monsoon			Fall			p-value[b]
			n	Median (IQR)	N > reg limit	n	Median (IQR)	N > reg limit	n	Median (IQR)	N > reg limit	
Chilies		30	18	12.20 (21.0)	2	16	16.02 (27.7)	4	18	16.83 (17.8)	5	0.75
Flour		15	13	1.38 (4.4)	1	13	9.79 (60.8)	6	20	2.51 (2.6)	0	*<0.01*
Groundnuts		10	1	0 (0)	0	6	57.51 (146.3)	3	12	0 (0.6)	2	*<0.05*
Maize		15	4	234.72 (597.0)	3	5	29.31 (24.2)	4	6	115.09 (239.8)	4	0.21
Pearl millet	AFB_1	15	8	5.82 (17.2)	2	10	55.64 (135.2)	7	10	0 (0)	0	*<0.01*
Rice		15	35	0 (2.8)	1	24	15.89 (49.8)	13	22	0 (0)	0	*<0.0001*
Sorghum		15	7	0 (3.0)	0	3	8.35 (63.5)	1	5	0 (0)	0	0.077
Wheat		15	12	0 (2.6)	1	12	71.45 (130.9)	7	13	1.83 (0.4)	0	*<0.0001*
Infant Formula		-	10	0 (0)	-							
Maize		2000[c]	4	5.70 (35.15)	0	5	33.36 (34.25)	0	6	0 (0)	0	0.29
Pearl Millet	FB_1	-	7	8.10 (22.58)	0	6	12.79 (17.63)	0	10	0 (0)	0	*0.015*
Sorghum		-	4	20.31 (19.45)	0	1	33.19 (0)	0	5	0 (0)	0	*0.0097*

[a]Regulatory limits set by the Food Safety and Standards Authority of India
[b]p-value for f-statistic (ANOVA) examining differences in [AFB_1] by season
[c]Regulatory limits set for raw maize grain by JECFA/FAO

Chapter 5: A Mixed-Methods Study of Pesticide Exposures in Breastmilk and Community & Lactating Women's Perspectives from Haryana, India

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Running Title: Pesticide contamination in breastmilk from Haryana, India

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Abstract

Background:

Population growth compounded by the effects of climate change and a need for increased crop yields to sustain food security have led to widespread use of chemical pesticides. The indiscriminate use of pesticides has in turn led to contamination of the environment, food commodities and bioaccumulation in human tissues, particularly in agrarian regions of India including the northern state of Haryana.

Methods:

We conducted a pilot screening study to investigate the presence of organochlorine, organophosphate, and pyrethroid pesticides in breastmilk samples (n = 75) from Haryana, India. Pesticide analyses were conducted using gas chromatography mass spectrometry (GC-MS) for OC and OP pesticides and GC-electron capture detector for pyrethroids. The study was complemented by a qualitative evaluation of maternal and community perceptions, knowledge, attitudes and practices associated with pesticide use and risk of exposure (n = 30 in-depth interviews; n = 9 focus group discussions).

Results:

Analysis of breastmilk showed the presence of *p,p'*-dichlorodiphenyltrichloroethane (DDT) and *p,p'*-dichlorodiphenyldichloroethylene (DDE) in 4% (range: <LOQ - 28 µg/L) and 5% (range: < LOQ – 107 µg/L) of samples, respectively. No other pesticides were detected.

Our qualitative findings showed that community members commonly held perceptions of pesticides as medicines and poison, but acknowledged their widespread use to ensure crop yields. Given the gendered engagement in farming in this setting, lactating women in study

communities do not directly handle chemical pesticides, thus lowering risk of inhalation and dermal exposure.

Conclusions:

In our small sample, breastmilk pesticide concentrations were low and did not pose a risk to infants. Based on the persistent nature of organic pollutants and reported widespread use, we recommend more comprehensive and longitudinal investigation of upstream pesticide contamination in the food supply and exposures among mothers and children.

Trial Registration: CTRI/2017/01/007636

Keywords: Organochlorines, Organophosphates, Pyrethroids, In-depth Interviews, Focus Groups

Highlights:

- We detected *p,p'*-DDT and *p,p'*-DDE in 4% (17.7 ± 8.9 µg/L) and 5% (46.9 ± 41.3 µg/L) of breastmilk samples
- Detected pesticide concentrations were low and likely do not pose a risk to infants
- While pregnant and lactating women have limited direct contact with pesticides, communities have concerns regarding the health effects of pesticide use

1. Background

It is estimated that in India, annual losses in agricultural production due to pests, average US$42.66 million [1]. Chemical pesticides are used to protect against such pests and include organochlorines (OCs) such as dichlorodiphenyltrichloroethane (*p,p'*-DDT) and hydrochlorocyclohexane (HCH), organophosphates (OPs), including chlorpyrifos and pyrethroids such as cypermethrin [2]. Pesticides can be used as insecticides, herbicides (to kill weeds), fungicides (to control mold) and as rodenticides (to control rats, mice and moles) [3]. The demand for OP pesticides such as chlorpyrifos, profenofos and malathion and synthetic pyrethroids such as cyfluthrin, cypermethrin, deltamethrin has increased in India [4]. Pesticide use in the Indian state of Haryana is amongst the highest in the country, at approximately 0.62 kg per hectare [1]. Studies in Haryana have shown that hardly 2-4 percent of farmers utilize masks, gloves, boots and other protective clothing during application of pesticides [5].

Organochlorine pesticides are also referred to as persistent organic pollutants (POPs). They bioaccumulate in the environment and become stored in adipose tissue due to their lipophilic, hydrophobic nature [6]. These pesticides exhibit low rates of chemical and biological degradation, accumulate and bio-magnify in the food chain [7]. OPs and pyrethroids are readily oxidized or hydrolyzed by CYP enzymes or hydrolases in the body. The biological half-life for most OP pesticides is measured in hours to days and, unlike OCs, these do not bio-accumulate appreciably [8]. The Stockholm Convention on persistent organic pollutants (POPs) recommended elimination of hazardous OCPs such as aldrin, dieldrin and has restricted the use of DDT. Hydrochlorocyclohexane (HCH) and its isomers were also recommended for elimination in 2009, due to emerging evidence of toxic effects on human health [9,10,11,12]. In

India, DDT and organophosphate pesticides continue to be used as household fumigants in vector control programs such as malaria [13].

Adverse health effects of pesticide exposure include cancer and in extreme cases, death [14]. Additionally, these xenobiotic pollutants can readily be transferred by inhalation or dermal exposure and through the food chain, to human breastmilk [15,16,17]. Infants may be exposed to pollutants through breastfeeding and they may become stored in the body depending on the molecular affinity of the chemical and body composition of the exposed individual [18,19]. Adverse effects of exposures to OC pesticides among children include cancer [20]. Exposure to OPs and pyrethroids have been linked to aberrations in cognitive, behavioral, sensory, and motor development in children [21,22,23]. Several studies have found pesticides in breastmilk samples from parts of India, although none have examined OCs, OPs and pyrethroid pesticides together, in the agrarian north Indian state of Haryana [24,25,26,27].

The overall objectives of this cross-sectional mixed-methods pilot study were to screen for and quantify levels of OC, OP, and pyrethroid pesticides in human milk samples from the Indian state of Haryana. Additionally, we aimed to examine community and lactating women's perceptions, knowledge, attitudes and practices (KAP) associated with farming and pesticide use.

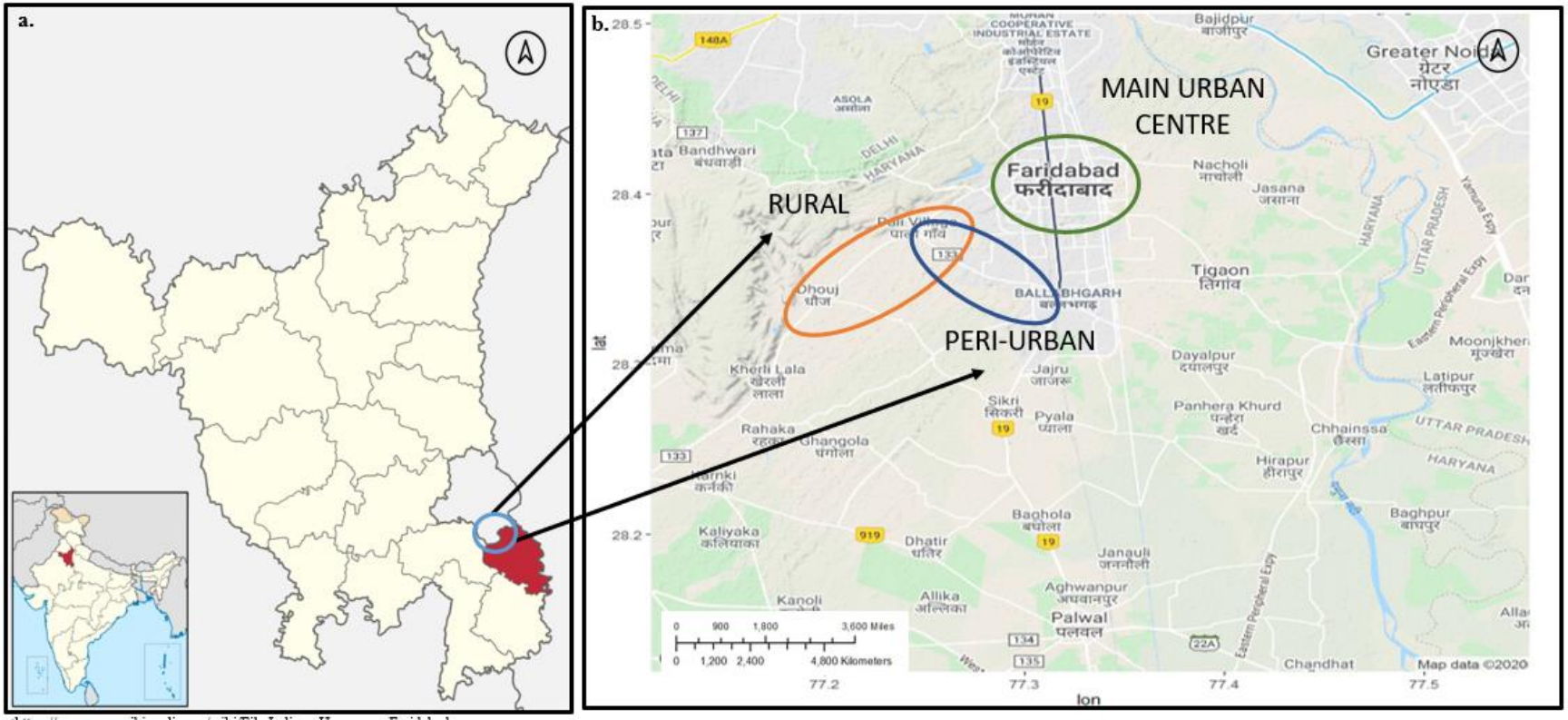
2. Material and Methods

2.1 Study population, mixed-methods data collection and analysis

This mixed-methods study involved the use of quantitative and qualitative methods to understand breastmilk pesticide exposures among women in rural and peri-urban Haryana. This work was part of a larger study which looked at maternal malnutrition and its impact on lactation performance (n = 232). Mothers between 18-45 years, with children 2-4 months of age, who were currently breastfeeding were enrolled in the parent study. Participants were recruited from

an existing surveillance database in rural and peri-urban parts of Faridabad district, Haryana (**Figure 1**).

Figure 1: Location map of rural and peri-urban study sites in Faridabad district, Haryana, India[a]



[a]https://commons.wikimedia.org/wiki/File:India_-_Haryana_-_Faridabad.svg

The study sites were chosen due to their proximity to New Delhi, the capital city of India. According to the National Family Health Survey – 4 (2015-2016), approximately 30% of children (< 5 years) in Faridabad district are stunted, 21% are underweight and 20% are wasted, and 14% of women (15-49 years) have a body mass index of < 18.5 kg/m^2.

Parent study exclusion criteria included women who were not currently breastfeeding their children, those who were consuming tobacco products, and those who were not likely to stay in the region for a period of 2 weeks post-enrollment. The study protocol involved contact with the family over a 14-day period, with information collected on socioeconomic status, demographic details, maternal morbidity and well-being, physical activity, water scarcity, food insecurity, infant morbidity and a 24-hour dietary recall. A wealth index was generated using principal component analysis (PCA) to determine tertiles of socioeconomic status and the Household Food Insecurity and Access Scale (HFIAS) was used to categorize households by food security status [28].

The study was approved by the Indian Council of Medical Research (ICMR), Society for Applied Studies Ethics Review Committee (ERC), and Emory University Institutional Review Board (IRB). Informed consent was read to all participants, who then provided written consent either using thumbprint or signature.

2.2 Human milk sample collection

Breastmilk samples were collected on day 1 post-enrollment from study participants. Mothers were asked to manually express full milk from one breast, until empty. Breastmilk was collected and stored at 2-8ºC. Breastmilk energy content and lipid concentrations were estimated using a crematocrit machine (Separation Technology, Inc.). Crematocrit was expressed as a percentage of the length of total milk column, varied with lipid concentration of milk. Stored

milk samples were kept at -80ºC and shipped to external laboratories for additional analyses. A stratified random sample of 75 breastmilk samples, between 5-7 for each month spanning the 12-month collection period were analyzed for pesticides. A total of 26 organochlorines, 15 organophosphates and 11 pyrethroids were assessed in human milk samples.

2.3 Chemicals

Magnesium sulfate (anhydrous), Primary Secondary Amine (PSA) and C-18 used for the extraction, were procured from Agilent Technologies (USA). Acetonitrile, acetic acid (glacial), and hexane (HPLC) were procured from Merck (India). Sodium acetate was procured from SRL (India). The PTFE syringe filters (0.45 um) used were purchased from Whatman (China).

2.3.1 Certified Reference Materials (CRM)

CRM for organochlorine pesticides comprised a mixture of 20 compounds viz. α-BHC , β-BHC, γ-BHC, Δ-BHC, heptachlor, Aldrin, heptachlor epoxide, *trans*-chlordane, *cis*-chlordane, endosulfan-I, endosulfan-II, endosulfan sulfate, 4,4'-DDE, 4,4'-DDD, 4,4'-DDT, dieldrin, endrin, endrin aldehyde, endrin ketone and methoxychlor; a herbicide mix of 5 compounds viz. atrazine, simazine, alachlor, metolachlor and butachlor and were procured from Sigma Aldrich (USA).

Individual standards for hexachlorobenzene, and several organophosphate pesticides including ethion, disulfoton, malaoxon, fenthion, phosalone, methyl parathion, methyl chlorpyrifos, ethyl parathion, fenitrothion, propazine and methyl paraoxon, were all procured from Sigma Aldrich (USA). Standards for malathion, acephate, phosphamidon, captafol, diazinon and monocrotophos were procured from Accustandard, Inc. (USA). A mixture of pyrethroid compounds including Cyfluthrin, L-cyhalothrin, cypermethrin-I, cypermethrin-II,

cypermethrin-III, deltamethrin, pendimethalin, permethrin-I, permethrin-II, fenvalerate, and tefluthrin were also purchased from Accustandard, Inc. (USA).

2.4 Breastmilk sample treatment

Pesticide extraction in milk samples was conducted using a modified and validated QuEChERS method, where 4 mL of milk was transferred to a 50 mL centrifuge tube to which a mixture of 3 g of magnesium sulfate, 0.5 g of sodium acetate and 10 mL of acidified acetonitrile (1% acetic acid in ACN) were added [8,29]. The contents were mixed vigorously by shaking for 1 min using a vortex mixer (Spinix, India) and centrifuged (high speed centrifuge machine: Remi, R-24, India) for 5 min at 8000 RPM. Next, 8 mL of the clear supernatant was transferred to a 15 mL centrifuge tube containing a clean-up mixture of 1 g magnesium sulfate, 200 mg Primary Secondary Amine (PSA) and 300 mg of C-18. The contents were vortexed for 2 min, then centrifuged for 5 min at 8000 RPM, following which 5 mL of the resulting supernatant was transferred to a test tube and evaporated to dryness, using a flash (Turbovap LV from Calliper Lifesciences – USA) evaporator under a stream of nitrogen. The residue was reconstituted to 1 mL of hexane and filtered through a 0.45 µm filter into a gas chromatography vial. The samples were further analyzed by GC-MS and GC-ECD.

2.5 Working standard solutions preparation

A stock standard mix of organochlorine pesticides and herbicides comprising a total of 26 compounds at 5000 µg/L concentration was prepared. Another stock standard mix comprising all organophosphate pesticides at 10,000 µg/L was prepared. The pyrethroid mixture was diluted to obtain a stock concentration of 20,000 µg/L. Working standards in the ranges of 10, 20, 40, 50, 75, 100 and 150 µg/L were prepared by following serial dilution techniques using hexane as the solvent and injected into GC-MS and GC-ECD to plot linear standard curves. Matrix matched

standards were used for quantification by preparation in bulk from cow's milk procured from different sources. Matrix matched blanks were checked to ensure lack of interference from any existing measured pesticides in our analysis. Matrix matched standards were prepared using blank matrix in the range of 10, 20, 30, 40, 50, 60 and 70 µg/L. These calibration curves were used for identification and quantification of pesticide residues in human milk samples.

2.6 GC-MS and GC-ECD analysis

Pesticide residue analysis was performed using GC-MS (Model: 7890B) and GC-Electron Capture Detector (Model 6890) from Agilent Technologies, USA. Chromatographic separation was achieved using an HP-5 MS capillary column (0.25 µm, 30 m x 250 µm, Agilent Technologies India Pvt. Ltd). The samples were injected using a multi-mode inlet (MMI) in spitless mode, with injector set at an initial temperature of 180ºC, ramped to 270ºC at 40ºC/min; with an injection volume of 2 µL. The ion source temperature was set at 270ºC. Oven temperature was programmed as follows: initial temperature at 80ºC held for 5 min, with a run time of 5 min and ramped at a rate of 10ºC/min to 200ºC with a run time of 17 min. Ramp 2 was set at 5ºC/min and temperature of 290ºC, where it was held for 2 min at a run time of 37 min and ramp 3 was set at 10ºC/min and temperature of 300ºC, held for 6 min with a total overall run time of 44 min. Mass spectrometer conditions included electron impact ionization of 70eV, a dwell time of 25 ms per ion transition, and a timed segment for Single Ion Monitoring (SIM) at 14^{th} and 25^{th} minute.

Pyrethroid pesticide residues were analyzed using optimized conditions in GC-ECD. Chromatographic separation was achieved using a HP-1 capillary column (0.25 µm, 30 m X 320 µm) from Agilent Technologies India Pvt. Ltd. Samples were injected in split mode, with an injection volume of 1µL, at isothermal temperature of 260ºC for a total run time of 25 min.

2.7 Quality control

All validation experiments were conducted using fresh cow's milk samples procured from local and commercial vendors, and served as controls. Samples were spiked with mixes of organochlorine (OC) pesticides, organophosphate (OP) pesticides at 10, 25 and 50 µg/L and pyrethroids at 50 µg/L to assess recoveries. Method specificity was also evaluated using fresh cow's milk from different vendors. Chromatograms were checked for the presence of interfering matrix signals and corresponding corrections made (if any) during interpretation of results. Three quality control levels were used, lower QCs at 10 µg/L, middle QC at 25 µg/L and higher QC at 50 µg/L. Identification and confirmation of detected pesticides in samples was done by comparing the retention times of sample peaks with that of standards and abundancy of qualifier ions in samples using MSD (µg/L) calculated as: calculated concentration (µg/L)*dilution factor/sample volume. In samples where pesticides were detected using GC-MS, further confirmation was conducted by analyzing the sample using alternate column and detector (ECD), calculated as:

*standard concentration (µg/L)*sample area*dilution factor/standard area*sample volume*.

Limits of detection (LODs), described as the lowest concentration at which an analyte can be detected in a matrix with a signal to noise ratio ≥ 3.0, were found to be 10 µg/L for OC and OP pesticides using MS; and 50 µg/L for pyrethroid pesticides using ECD. Limits of quantification (LOQs), describe concentration at which S/N ratio for quantifier ions ≥ 10 when comparing analytes from matrix matched calibration standards. For pesticides these ranged from 25 µg/L for OC and OP pesticides to 50 µg/L for pyrethroids.

Recoveries for all pesticides were within the acceptable range of 70-120%, with the exception of compounds including acephate, and monocrotophos which had relatively lower

recoveries (25%) at lower QC levels (25 µg/L) [30]. All pesticide standard curve concentrations ranged between 10–150 µg/L with R^2 values between 0.997-0.999, and matrix matched calibration curves had concentrations ranging from 10-70 µg/L with R^2 values between 0.991-0.998.

2.8 Qualitative data collection

To complement breastmilk pesticide analyses, a qualitative study was conducted between April and May 2018 to understand community perceptions, knowledge, attitudes and practices associated with farming and risk of pesticide exposures. We used in-depth interviews (IDIs) and focus group discussions (FGDs) to elicit information on perceptions of food systems and environmental exposures. We collected information on a number of thematic areas, spanning the household food chain with a focus on homestead production, farming and use of pesticides and fertilizers. Study sites for the qualitative component (n = 10) were chosen purposefully and stratified to represent rural (n = 5) and peri-urban (n = 5) communities and to ensure representation of Hindu and Muslim households. In these communities, we conducted a total of 30 IDIs with lactating women who had children < 7 months of age and who were previously enrolled in the parent study. Of these, 14 IDIs were conducted with women from peri-urban communities, which in this region are located closer to Faridabad city. Most households in peri-urban communities do not have access to farmland nor do they practice farming. The remaining 16 IDIs were conducted with women from rural communities, all of whom owned or worked on farmland and came from livestock owning households. Rural communities sampled for this study are less densely populated and located further away from Faridabad city.

We also conducted a total of nine focus group discussions across three communities. In each community, we conducted one FGD with each of the following groups: mothers-in-law and

elder women, elder men and fathers and frontline workers (FLWs). Frontline workers included Accredited Social Health Activists (ASHAs) and Aanganwadi Workers (AWWs), functionaries of the National Health Mission and Integrated Child Development Scheme, respectively.

Prior to commencing interviews, an interview guide was developed and included sections corresponding to household food production and associated chemical use. Participants were interviewed by the first author in their households. The qualitative nature of this work, particularly within the cultural context of India, necessitated in many instances, that other members of the household be present. Where feasible, the interviewer requested that other members, particularly male family members excuse themselves to allow women to express themselves freely. Interviews were conducted until a saturation of themes was reached. We employed an emic approach, whereby the guide was revised and enhanced based on themes arising during the course of fieldwork. Each interview took approximately one hour, and participant fatigue and burden were key considerations throughout.

For community member focus group discussions, we enlisted the assistance of community elders in target communities to assemble mothers-in-law, fathers-in-law, fathers and FLWs. To avoid intrusion on work schedules, we spoke with FLWs beforehand and asked them to assemble at a day and time that did not conflict with their work responsibilities.

Detailed observations were recorded throughout the process, both in the context of household visits and community interactions. Several ethnographic tools were employed over the course of this work, including active listening [31], observations and note taking, formal and informal interviewing and critical self-reflection [32]. All IDIs and FGDs were audio recorded after acquiring consent and were transcribed verbatim from Hindi/Haryanvi to English. Transcriptions conducted by a trained Hindi-English translator were verified by the first author.

All observations and reflections were recorded as field notes, which were also documented in Microsoft Word on a daily basis during fieldwork.

A codebook was developed employing both *inductive* themes, which arose from respondents during interviews and *deductive* themes, which were identified based on the literature and while developing the interview guides [33]. Transcripts were open-coded manually using MAXQda (18.2.0, GmbH, 2018) and 10% were double-coded by an independent second coder to ensure inter-coder reliability. Thematic analysis was employed to analyze the data [34,35]. We applied a knowledge, attitudes, practices (KAP) framework to our analysis of farming practices, food production habits and pesticide use among community members.

3. Results

3.1 Participant demographics and pesticide residues in breastmilk

Demographic characteristics of women for whom breastmilk pesticide residues were analyzed and their children are presented in **Table 1**.

Table 1: Household, maternal and child characteristics of N = 75 participants in Haryana, India

Household Characteristics	**N (%)**
Religion	
Hindu	51 (68)
Muslim	23 (31)
Other	1 (1)
Caste	
Scheduled caste	20 (27)
Other backward caste (OBC)	34 (45)
Other caste	21 (28)
Paternal Occupation	
Not working/retired/unemployed	4 (5)
Business/petty trader/self-employed	16 (21)
Salaried employee	38 (51)
Other	17 (23)
Socio-economic Status	
Low	25 (33)
Middle	25 (33)
High	25 (33)
Residence	
Peri-urban	58 (77)
Rural	17 (23)
Food Insecurity	
Food secure	62 (83)
Food insecure	13 (17)
Maternal Characteristics	**Mean (SD)/N (%)**
Maternal Age (years)	25.1 (3.75)
Maternal Education (years)	6.6 (5.3)
Parity	2.3 (1.3)
Anthropometry	
Weight, kg	60.0 (10.2)
Height, cm	152.3 (5.9)
BMI, kg/m^2	21.9 (3.7)
BMI categories, kg/m^2	
Underweight (< 18.5)	10 (13)
Normal (18.5-25)	52 (69)
Overweight (25-30)	11 (15)
Obese (> 30)	2 (3)
Human milk fat content (g/L)	38.8 (15.6)
Current Dietary Patterns	**N (%)**
Vegetarian	29 (39)
Non-vegetarian	40 (53)
Eggetarian	6 (8)

Child Characteristics	N (%)
Child Sex	
% Male	33 (44)
% Female	42 (56)
Child Age (months)	3.7 (0.60)
Child Nutritional Status	
% Stunted (length-for-age z-score < -2)	14 (19)
% Underweight (weight-for-age z-score < -2)	12 (16)
% Wasted (weight-for-length z-score < -2)	5 (7)
Current breastfeeding practices	
Currently exclusively breastfeeding	25 (33)

Average age (± SD) of women was 25.1 ± 3.75 years and median parity was 2.0. Maternal breastmilk fat content (38.8 ± 15.6 g/L) values are consistent with previous studies on human milk persistent organic pollutants (POPs) [25,36,37]. A total of 53 OCs, OPs and pyrethroids were analyzed in this study. We detected *p.p'*-DDT in three (4%) samples and *p,p'*-DDE in four (5%) samples. *p,p'*-DDT was detected in two samples at levels < limit of quantification (25 µg/L) and in one sample at 28 µg/L; *p,p'*-DDE was detected at levels of 34, 31, 107 µg/L and < LOQ in the fourth.

One sample in our study exceeded maximum residue levels (MRL) of 0.05 mg/kg set by the FAO/WHO, for both DDT (28 µg/L) and DDE (107 µg/L) in milk. *p,p'*-DDE is a marker of past exposure to DDT and its primary degradant [38,39]. We calculated the ratio of DDT:DDE in the aforementioned sample to be ~ 1:4 or 0.26, indicating DDT exposure was recent (ratio <5 indicates recent or ongoing exposure) [36]. This ratio decreases over time as DDT degrades [38,39]. No other pesticides were detected in samples from our study. Three of the four women for whom *p,p'*-DDE was detected were from peri-urban communities, the fourth from a rural community. Three women were primiparous and one was multiparous (2 children).

3.2 Qualitative Study of Household Food Production: Knowledge, Attitudes & Practices related to Pesticides and Fertilizers

Demographic details of participants of in-depth interviews and focus group discussions are presented in **Table 2**.

Table 2: Demographic characteristics of in-depth interviews and focus group discussion participants

Population	**Peri-urban**	**Rural**
In-depth Interviews (n = 30)		
Lactating Mothers (#)	14	16
Maternal age (mean)	26.3	23.9
Religion (n)	14 – Hindu	8 – Hindu, 8 - Muslim
Focus Group Discussions (n = 9)		
Mothers in-law/Women (#)	6	16
Age (mean)	45.7	59.2
Religion (n)	6 – Hindu	10 – Hindu, 6 – Muslim
Fathers in-law/Fathers (#)	7	17
Age (mean)	52.4	47.5
Religion (n)	7 – Hindu	8 – Hindu, 9 - Muslim
Frontline Workers (#)	5	12
ASHAs	-	6
AWWs	5	6
Age (mean)	36	32.9
Religion (n)	5 - Hindu	11 – Hindu, 1 - Muslim

Approximately 30% of women in our sample practiced farming in their current residence and 43% had historically practiced farming in their pre-marital households. Families in our study communities owned small plots of land, averaging approximately 2 acres. Main crops farmed in these communities include wheat, sorghum, pearl millet, animal feed for buffalo, and mustard seeds. A few households noted growing rice and maize. Most households practiced subsistence farming, with a few producing enough wheat for commercial sale. Primary irrigation facilities for farming come from groundwater borewells and pipes. Seeds for cultivation are primarily procured from the market, however some families described using seeds planted in previous

seasons, after traditional preparation. Soil is cultivated using tractors and other mechanized equipment.

The results of the analysis and interpretation of our qualitative data are presented as key themes identified with respect to the research question: What are *perceptions* of and *knowledge*, *attitudes* and *practices* associated with farming and pesticide handling and use among community members and lactating women in Faridabad, Haryana? The following themes emerged with regards to community and lactating women's perceptions of, knowledge, attitudes and practices associated with pesticides and fertilizers:

- Perceptions and attitudes towards pesticides as medicine vs. poison and perceived health effects of pesticides and fertilizers
- Knowledge and practices related to pesticide handling and use
- Gendered engagement in use of pesticides and fertilizers

Perceptions and attitudes towards pesticides as medicine vs. poison and perceived health effects of pesticides and fertilizers

During focus group discussions, several community members across rural and peri-urban sites described pesticides as "*medicines for crops*" as well as "*poison*". Similar perspectives were held for fertilizers. These dichotomous views of pesticides and fertilizers informed an understanding of the benefits and harms of such chemicals and community members' perceptions and attitudes towards them. They noted that such chemicals are essential to ensure adequate yield of crops and hence necessary to avoid hunger. Yet, people were torn between the benefits of pesticides for adequate crop yield and their potential and perceived deleterious effects on humans.

"To protect the crops from insects otherwise the insects will infect the roots and the crops will die. Nothing happens it only kills the insects in the wheat and once we eat we wash it so there is no effect."

- FGD with mothers-in-law (peri-urban)

"Four years later, when more money came we started using Sipra, which has made the crops poisonous. Because this medicine is poisonous, if we eat it, it can cause problems."

- FGD with mothers-in-law (rural)

Despite this recognition, there was an overall sense of futility at a lack of alternatives or options.

"These medicines are all poison, even then we are compelled to use."

- FGD with mothers-in-law (rural)

With regards to perceived health effects, most community members were wary of pesticide and fertilizer use and their implications on human health. Some community members noted that children's height remains "short" because of exposure to chemicals. Community members noted that chemicals can be transferred from maternal diet and breastmilk to children, which can in turn adversely affect their health and well-being.

"It affects the lactating mother, we put potash for growth and mother feeds milk to their child to strengthen bones of the child. But if the mother will eat that food then it will go to the child as well. We put poison in the crops then it will affect the body, it weakens the body."

- FGD with fathers-in-law (rural)

"One can become unconscious and suffer from headache. Diseases can occur, children can be aborted. I put the medicines so that we can eat. Because of these tablets and medicines, diseases are occurring. Some are getting appendicitis, hernia. What to do in

that, nothing can be done, we give rice and milk to eat. If the boy is small and girl is tall then they will not get good match for getting married."

- FGD with fathers-in-law (peri-urban)

"There is no option. To fill the stomach and for the growth of cereals, urea needs to be added. It is affecting the growth of the children. Whatever mother and father are eating, children are also eating that only. Now 5-year-old children are not growing properly. Urea and manure are not proper now a days."

- FGD with fathers-in-law (rural)

Community members also described perceptions of the effects of pesticide and fertilizer use on the overall health of the population, and their role in chronic diseases such as cancer and obesity.

"Maximum occurrence is there for cancer, TB, everything is happening because of these chemicals, weak eye sight, obesity"

- FGD with mothers-in-law (rural)

"Buffalo eats fodder and the fodder contains urea and milk will be formed. From that only urea is put in complete farm and all the eatables contain urea. When government is selling such things then they should sell the products that are beneficial, in place of urea they can give us something else. Acidity, blood pressure, heart attack as it has direct effect on the heart, if more urea is put then it has worse effect. They are putting more urea as knowledge is not there, proper training is not there."

- FGD with fathers-in-law (peri-urban)

As with pesticides, there remains an understanding of the possible ill-effects of excess fertilizer use and consumption, and a simultaneous sense of dependence on these chemicals to

ensure crop health and adequate yields. Elder community members were able to describe a transition to use of modern-day synthetic chemicals as replacements for natural manure. They cited that the natural products (i.e. cow dung) are healthier for humans but not effective at producing adequate crop yields.

Knowledge and practices related to pesticide handling and use

Both elder and lactating women during FGDs and IDIs, described the use of "*keetanashak dawa*" or insecticides and "*kharpatwar*" in order to kill weeds. When probed regarding their knowledge including names of specific pesticides used, women were able to identify DDT and Sipra, a powder which is sprayed on crops. Some elder women were aware of regulations around procurement of chemicals.

> *"We do not get medicines just like that, before they give us, they verify how much land is there, that much medicine only they will issue. If you ask for 10 acres of land and you have only 2 acres then you won't get medicine for 10 acres.*
>
> - FGD with mothers-in-law (rural)

Pesticide spraying is common practice in study communities, however limited if any personal protective equipment (PPE) such as masks and gloves are used and can include a cloth which is tied over the mouth so the spray isn't inhaled. Additionally, slippers or shoes are worn on feet. When questioned about the purpose and intention behind the use of pesticides, communities described issues with insect infestation, namely "*Siroli* or *termites*", and noted that "medicines" are used to protect crops from pests.

> *"Fungal infection and insects do not harm the crops and they grow healthy, it protects the crops from being destroyed."*
>
> – HH IDI with lactating woman (rural)

They also noted the use of "*dung and manure*" to protect crops from insect infestation and related damage, specifically identifying dialkylphosphates (DAP), zinc and urea. Like pesticides, fertilizers are generally applied to fields, manually, with bare hands or using gloves, with limited PPE. Most women had little knowledge of the exact location from where pesticides are procured and noted that they are stored away from the household, on the farm, where children and other household members cannot access them.

Additionally, women noted that the chemicals are disposed of, away from the household. Several community members and women were aware of incidences where these chemicals had been used for human consumption, leading to death. This created a general reticence around their handling, storage and use, with the task being handled primarily by elder males within the household.

> *"After spraying the medicine, it is thrown in the jungle along with its tumbler. Whenever it has to be sprayed, it is brought out, otherwise it is not brought out. If a fight happens, in anger somebody may eat it and die. No one keeps it in their house, farm doctor gives this medicine and it is stored on the farm."*
>
> – HH IDI with lactating woman (rural)

Gendered engagement in farming and use of pesticides and chemicals

Cultural norms, particularly in some Hindu communities within this region dictate that younger women do not leave the home. Although lactating women in several communities do go out to farm, when other community members were asked, they noted that pregnant women do not engage in farming as they risk "aborting" their child. Additionally, community members noted that many women do not farm due to personal safety concerns.

> *"Like this also daughters and daughter in laws do not go to the farm, there is no safety"*
>
> - FGD with mothers-in-law (rural)

Women in rural Muslim households do engage in farming activities, which primarily include watering the soil and harvesting, described as "*crop cutting*". This is likely due to disparities in socioeconomic status, where households within these communities may not be able to afford outside labor, thus necessitating women's engagement. Although collectively, farming remains the domain of male members of the household in study communities.

"No, in our house ladies are not aware about farming. Gents are aware about farming"

- HH IDI with lactating women (peri-urban)

It was noted that lactating women are not directly involved in the handling and use of pesticides.

"No, it does not affect us as we do not spray the medicine, it is sprayed by some other person"

– HH IDI with lactating woman (rural)

Rather, most women noted that their families hire outside labor to spray pesticides using machines, though their husbands or fathers-in-law also engaged in pesticide spraying. As a result of their limited engagement in farming and pesticide handling, lactating women's knowledge of pesticides was rudimentary.

4. Discussion

This pilot study was undertaken to detect pesticides in a cross-sectional sample of human breastmilk, and to understand knowledge, attitudes and practices of lactating women and communities with regards to pesticide and fertilizer use. We were able to detect DDT and DDE in a small percentage of samples in our study. No other pesticides were detected. Prior studies conducted in regions in close proximity to our study site, including New Delhi, the national capital of India have found organochlorine pesticide concentrations in human breastmilk samples

at levels between 15.8 ± 8.48 µg/L for total-Hexachlorocyclohexane (HCH), 1.8 ± 1.12 µg/L for DDT and 2.9 ± 2.54 µg/L for DDE [24]. Bedi et al., (2013) reported mean ΣDDT (DDT + DDE) concentrations in breastmilk of 1.914 µg/L lipid weight, collected from the state of Punjab [25].

Another study conducted in the north Indian state of Punjab found mean breastmilk concentrations of *p,p'*-DDE at 0.00056 ± 0.0042 µg/L [26]. More recently, Bawa et al., (2018) found *p,p'*-DDE at 0.407 ± 0.885 and 0.345 ± 0.815 µg/L lipid weight from the north Indian cities of Bathinda and Ludhiana [27]. They also detected ΣDDT at 0.519 ± 1.017 and 0.415 ± 0.846 µg/L lipid weight in both cities respectively [27].

Mean values for the samples where DDT (17.7 ± 8.9 µg/L, N = 3) and DDE (46.1 ± 41.7 µg/L, N = 4) were detected in this study are slightly higher. The limit of detection (LOD) in our screening study is also higher (10 µg/L; LOQ of 25 µg/L for OCs) than those in the published literature from India, which range between 0.008-0.02 µg/L [27]; 0.001 µg/L (OCPs) and 0.002 µg/L (OPs and SPs) [26]. It is important to note that our LODs (10 µg/L) were below maximum residue levels of 0.05 mg/kg (50 µg/L) set by the FAO/WHO for DDTs in milk, thus allowing us to detect trace concentrations and values of public health significance [38]. Based on our finding, it is possible that trace levels of other pesticides were not detected, but are present in breastmilk. In addition, it is also likely that the gradual phasing out of these compounds in India has resulted in lower concentrations of residues in breastmilk.

Studies of pesticides in breastmilk from other countries have reported DDTs at concentrations ranging between 0.119 µg/L fat in Japan [40] to 3.56 µg/L fat in Iran [41]. In Australia, Du et al., (2018), found ΣDDT at 0.063 µg/L fat for samples collected between 2013-2015 [37]. LODs and LOQs for DDT and its metabolites in these studies ranged between 0.2-0.5 µg/L and 0.5-1.0 µg/L respectively.

Approximately 90% of exposure to pesticides occurs via dietary intake as opposed to dermal or inhalation routes [42]. Several studies conducted in northern India have found residues of DDT, DDE, and other organochlorine pesticides in various staple crops such as rice (median value of 0.01 mg/kg; 90% of samples > MRL of 0.1 mg/kg) and produce including cauliflower, tomatoes, raddish, brinjal, spinach among others [43,44,45]. Testing food samples in addition to breastmilk is recommended for future studies.

Based on our qualitative findings, lactating women in study communities do not engage directly in pesticide spraying and use. That does not preclude secondary inhalation, ingestion or dermal routes of exposure. For women who do engage in farming, training on integrated pest management practices and additional agricultural extension services may be warranted [46].

Concerns around the consumption of pesticides to induce suicide, described by women and community members in our study have been reported elsewhere in India [47]. Several participants described “means restriction” or limiting access to pesticides to avoid potential self-harm, as an important driver for storage of chemicals away from household [48]. This in turn, safe guards lactating women and children from direct exposure to harmful pesticides. Dichotomous perceptions of pesticides as “medicines” and “poison” among community members, do not prevent their use. There is a recognition that such chemicals are necessary to ensure adequate crop yields, despite potential harm. For peri-urban samples in the study, it is possible that women have had historical exposure to POPs in their pre-marital households, where farming is practiced. The study of long-term and intergenerational risk of exposure using longitudinal study designs, with larger sample sizes is therefore warranted.

In addition to pesticides, further research is needed to understand the implications of extensive fertilizer use in study communities, with concomitant surveys of biomarkers of

exposure in human samples. Community members equated exposure to chemical fertilizers such as urea and potash with several deleterious health effects and stunting in children. More research is needed to validate these concerns while accounting for the essential nature of such chemicals in maintenance of crop yields and to ensure food security.

5. Conclusions

Our study suggests a need for further investigation of food systems and environmental exposures including pesticides and fertilizers, in peri-urban and rural communities of Haryana, India. Future studies should be conducted in more agrarian regions of the state and in remote and rural communities where risk of exposures is higher. Those most vulnerable to excessive pesticide and fertilizer use and consumption through any and all pathways and routes should be sampled to ensure true representativeness and generalizability of exposure assessment findings.

Although pesticides do not appear to be a major concern in breastmilk in this small sample, it is prudent to investigate their presence upstream in the food supply chain. As women do not engage directly in farming, their risk of direct occupational exposure remains low. As noted above, these findings should be considered only within the context of the existing sample and population studied. Further work is needed to understand how our findings compare to communities in more remote and rural regions of Haryana, with varied caste, religious and occupational patterns.

Exclusive breastfeeding is recommended for children for children < 6 months of age, and breastmilk remains an essential source of nutrients and immune factors for growing babies. Based on our findings, risk of pesticide exposure for children via breastmilk is not a major concern in this population, however testing of pesticide residues in animal milk, dairy products,

complementary and weaning foods is recommended based on results from other studies in the region [4,49].

List of Abbreviations:

DDE: Dichlorodiphenyldichloroethylene

DDT: Dichlorodiphenyltrichloroethane

LOD: Limit of Detection

LOQ: Limit of Quantification

MRL: Maximum Residue Limits

OCs: Organochlorine Pesticides

OPs: Organophosphate Pesticides

POPs: Persistent Organic Pollutants

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Declarations:

Ethics Approval and Consent to Participate:

Ethical approval for this study was acquired from the Indian Council for Medical Research (ICMR) Health Ministry's Screening Committee (HMSC), Society for Applied Studies Ethics Review Board (ERB) and Emory University Institutional Review Board (IRB). Informed consent was read to each participant in the study and written consent or a thumb print was acquired.

Consent for publication:

Not applicable

Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

RVM and MFY designed the study. ST and SR collected all breastmilk samples. Sample analysis was conducted by SMA and MM. All qualitative data was collected and analyzed by RVM. RVM drafted the manuscript. AWG, UR, PBR, RM and MFY critically reviewed the manuscript. RVM, MFY, RM procured funding for the study. All authors have read and approved the final version of this manuscript.

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Chapter 6: Mediation path analyses of associations between breastmilk mycotoxins and infant growth outcomes

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Statement: *This chapter reflects an exploratory learning objective. We did not have adequate sample sizes and were not powered to detect effect sizes.*

Abstract:

Exposure to mycotoxins has been associated with impaired gut integrity and adverse growth outcomes in infants. We conducted an exploratory mediation path analysis to understand potential pathways of associations between aflatoxins in human breastmilk samples, breastmilk inflammatory markers, infant gut integrity and infant growth faltering. We were able to detect and quantify 6 aflatoxins (B_1 + B_2, G_1 + G_2 + M_1 + M_2) in (n = 100) breastmilk samples at cumulative concentrations ranging between 70.2-1494.80 pg/mL (mean: 137.25 ± 164.88 pg/mL). Several inflammatory markers were also quantified in breastmilk samples including IL-8, TNF-α, sIgA and lactoferrin. Infant gut integrity was measured using lactulose mannitol ratio in urine (mean: 0.056 ± 0.086). Only 2 infants in our sample showed problematic L:M (≥ 0.15), indicating compromised gut integrity. Finally, several indicators of child nutrition outcomes including length-for-age, weight-for-age and weight-for-length z-scores were also tabulated.

Results of linear (LAZ, WAZ, WFL as outcomes) and logistic (stunted, wasted, underweight as outcome) regression analyses in bivariate and multivariable adjusted models did not show significant associations between individual predictors and mediators in exposure path models. Although model fit indices showed good fit, none of the direct or indirect effects in our mediation analyses were significant. While, this study was not designed or powered to examine these relationships, biological plausibility has been extensively studied using *in vivo* and *in vitro* models and these studies suggest impacts of mycotoxin exposure on gut integrity and growth. Although our analyses did not show significant effects, further studies with larger sample sizes are warranted.

1. Introduction

High levels of child malnutrition in India in the first 6 months of life represent a critical public health challenge. Exclusive breastfeeding during this time period is one of the most cost-effective child survival interventions available today; however, gaps remain in our knowledge on how to prevent wasting or stunting in exclusively breastfed children. Nutrition is an established factor contributing to early growth faltering, but a major unanswered research question is the contribution of environmental exposures (including mycotoxins and pesticides) to growth failure. These exposures may contribute to environmental enteric dysfunction (EED), a sub-clinical inflammatory condition that results in a leaky gut, malabsorption of essential nutrients and child malnutrition (Owino et al., 2016; Mapesa et al., 2016). Studies conducted to date suggest that mycotoxin exposure leads to impaired growth and immunity, mediated by EED. Mechanisms of action include reduced protein synthesis, inhibition of sphingolipid production and increased production of pro-inflammatory cytokines and decreased appetite. To date, no study has fully investigated the pathway of environmental exposure from breastmilk mycotoxins to infant growth faltering, mediated by breastmilk inflammation and impaired infant gut integrity. We hypothesized the following: *[↑Breastmilk Mycotoxins] → ↑ breastmilk inflammation → ↓ infant gut integrity → child growth faltering*.

We examined associations between breastmilk mycotoxins, namely *ΣAflatoxins*, breastmilk inflammatory markers, namely *IL-8, TNF-α*, *sIgA* and *lactoferrin*, infant gut integrity and infant growth outcomes. These preliminary analyses were conducted to investigate whether concentrations of breastmilk mycotoxins are associated with infant gut integrity and growth, mediated by an inflammatory breastmilk profile. As presented in chapter 4, 8 mycotoxins were examined in n = 100 breastmilk samples from women with children 2-4 months of age in India.

Aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2) and Ochratoxins (A and B) were examined, and several AFs were detected in breastmilk samples in our study.

Several markers of inflammation were also quantified in human breastmilk samples. Of these, IL-8 and TNF-α are proinflammatory cytokines. IL-8 is essential for recruitment of neutrophils and works to enhance intestinal development in infants (Ballard & Morrow, 2013). Additionally, IL-8 protects against TNF-α mediated intestinal damage. Higher levels of IL-8 are seen in breastmilk under conditions of mastitis, or mammary inflammation. IL-8 is also responsible for recruitment of neutrophils (Ballard & Morrow, 2013). TNF-α on the other hand, stimulates inflammatory immune activation.

The next group of inflammatory markers are antibodies for antigens, of which secretory immunoglobulin A (sIgA) is the most predominant antibody in human milk. sIgA is a protein and inhibits the binding of pathogens. It produces maternal specific immunoglobulins to environmental antigens (Ballard & Morrow, 2013; Wagner et al., 1996). We also quantified levels of lactoferrin in breastmilk samples. Lactoferrin is an acute phase protein responsible for chelation of iron, it is also anti-bacterial and an anti-oxidant. It is a bacteriostatic, iron-binding protein with additional antiviral properties (Mead, 2008; Wagner et al., 1996).

We also measured sodium potassium ratio to characterize sub-clinical mammary inflammation. The Na:K ratio in breastmilk has been associated with higher levels of IL-8, sIgA and lactoferrin markers of inflammation. Inflammation associated with sub-clinical mastitis results from elevated production of proinflammatory cytokines and chemokines (Tuaillon et al., 2016; Li et al., 2017). SCM also triggers physical damage to mammary tissue, results in opening of tight junctions and increased mammary epithelium permeability, which enables paracellular transport of sodium (Li et al., 2017).

Pro-inflammatory cytokines that are produced during the inflammatory response include TNF-α, interferon-γ (IFN-γ), interleukin-1β (IL-1β) and IL-12, which in turn cause disruptions to the intestinal tight junction barrier and result in increased tight junction permeability (Al-Sadi et al., 2013). Pro-inflammatory cytokine induced increase in intestinal tight junction permeability is an important pathogenic mechanism contributing to increased intestinal inflammation. Anti-inflammatory cytokines which include IL-10 and TGF-β protect against disruption of intestinal tight junction barrier and development of intestinal inflammation (Al-Sadi et al., 2013).

Urinary lactulose mannitol ratios were used as a biomarker for infant gut integrity. Intestinal permeability is altered in many pathological states including inflammatory bowel disease, celiac disease, cirrhosis, sepsis and environmental enteropathy (Travis et al., 1992; Bjarnason et al., 1995). Undernutrition may cause abnormalities in intestinal permeability, which in turn is implicated as a cause of growth faltering among children living in poverty. Saccharide probes are routinely used to assess gut integrity, particularly in field settings (Kelly et al., 2004; Lunn et al., 1991; Campbell et al., 2003). Probe sugars such as lactulose and mannitol become passively absorbed from the gut, are minimally metabolized and excreted in urine in proportion to the administered dose. Combined saccharide solutions include a smaller and more permeable mannitol, which is a monosaccharide with a maximum radius of 0.38 nm and molecular weight of 182 Da, polar surface area of 121 Angstrom^2. The larger disaccharide, lactulose has a molecular radius of 0.62 nm, weight of 342 Da and a polar surface area of 190 A^2.

Transport of hydrophilic mannitol occurs via 2 distinct pores formed by tight junction proteins which include claudins and occludins, of diameter 0.5 and 1 nm, respectively (Linnankoski et al., 2010). Decreased mannitol recovery reflects damage to the collective villous surface area. Lactulose recovery reflects permeability to large molecules that are likely to be

concentrated in crypts (Menard et al., 2010). L:M ratio has been associated with growth faltering among at-risk infants across several studies (Lunn et al., 1991; Panter-Brick et al., 2009; Weisz et al., 2012).

The purpose of the exploratory investigation presented in this chapter was to understand how to develop models to explore associations between mycotoxins in breastmilk and infant growth faltering We hypothesized that exposure to breastmilk mycotoxins will be associated with infant growth faltering, mediated by an inflammatory breastmilk profile and impaired infant gut integrity. As an **important caveat,** the preliminary analyses presented in this chapter were conducted as a learning exercise. We were limited by a small sample size and were not powered to detect associations, due to post-hoc additions of these secondary research questions to the parent study. These analyses were conducted as a potential proof-of-concept, and an extension of our hypotheses about the associations of environmental exposures with early infant growth outcomes.

2. Materials & Methods

1) Breastmilk Mycotoxins

Methods for breastmilk mycotoxin analyses are presented in chapters 3 and 4. Briefly, 8 mycotoxins were analyzed using ultra-high-performance liquid chromatography tandem mass spectrometry. All breastmilk mycotoxins were conducted at the *Food Safety and Analytical Quality Control Laboratory, CSIR-Central Food Technological Research Institute*, Mysuru, India. All breastmilk inflammatory marker, lactulose mannitol analyses were conducted at the *St. John's Research Institute*, Bengaluru, India. Infant anthropometry was measured during field visits to participant households in Haryana, India, as per protocols described in chapters 3 and 4, by the CHRD-Society for Applied Studies team.

2) Breastmilk Inflammatory Markers

***Interleukin-8, TNF- α, sIgA, Lactoferrin*:** IL-8 (TNF- α, sIgA, lactoferrin) estimation was conducted using a sandwich enzyme-linked immuno-sorbent assay, Abbexa kit. Anti-IL-8 was coated onto a 96-well plate. Biotin conjugated anti-IL-8 antibodies were used as detection antibodies. Standards, test samples and biotin conjugated antibodies were then added to the wells and washed with wash buffer. Avidin-biotin-peroxidase complex was then added and unbound conjugates were washed away with wash buffer. TMB substrate was used to visualize HRP enzymatic reaction. TMP was catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. Intensity of the yellow color is proportional to IL-8 amount bound on the plate. O.D. absorbance was measured spectrophotometrically at 450 nm in a microplate reader, and concentration of IL-8 was calculated against the standard graph plotted. Thawed breastmilk samples were centrifuged in a cooling centrifuge at 10,000 RPM for 20 min. The supernatant (100 µL) was used for determination of IL-8 concentrations. (For the lactoferrin assay, the samples were diluted 1000 x with milliQ water). In-house pooled samples were used for quality control purposes. The inter-assay and intra-assay CVs were < 8% and 10% respectively.

***Sodium:Potassium*:** Na was measured using flame photometry method (Chemi Line, Digital Flame Photometer, CL 410, Labline Technologies Pvt. Ltd). The principle behind this method is that trace amounts of sodium can be determined by direct reading type of flame photometer. The sample is sprayed into a gas mixture flame and excitation is carried out under carefully controlled and reproducible conditions. Light intensity is measured by a phototube. The intensity of the light is proportional to the concentration of the element.

Sodium stock solutions were prepared using 2.542 g of NaCl which was dried at 140ºC and dissolved in 1000 mL distilled water, 1 mL = 1 mg of Na. Working standards were prepared at concentrations of 10, 20, 30, 40, 50 mg/L by diluting 1, 2, 3, 4, and 5 mL of stock Na solution to 100 mL using distilled water. The sodium filter was selected with the help of filter selector of the burner unit of the flame photometer. The burner was ignited and the air supply pressure was adjusted between 0.4-0.6 kg/cm^2, as well as the gas supply to get a blue cone flame in the burner. Distilled water was aspirated to the atomizer, after 30 seconds, the meter reading was adjusted to 0. The standard solutions were first aspirated to adjust meter reading to 50 by using 50 mg/L standard solution. Next, 20 mg/L standard solution was aspirated, taking care to run distilled water between standard runs and ensuring that the meter shows zero reading. The samples were thawed, mixed thoroughly and were run after appropriate dilution. A calibration curve was plotted using the standard readings and the sodium concentration for samples were calculated using the standard curve. In house pooled breastmilk samples were used for quality control purposes. The inter-and intra assay CVs were 2.5% and 1.3% respectively.

Potassium was measured using the same method described for Na, with some modifications. Here, samples were sprayed into an air-fuel mixture flame and excitation was carried out under carefully controlled reproducible conditions. The intensity of emitted light was measured using a phototube. The intensity of light is proportional to the concentration of the element. K filter was selected with the help of filter selector of the burner unit of the flame photometer. The burner was ignited and the air supply pressure was adjusted between 0.4-0.6 kg/cm^2 and gas supply so as to get a blue cone flame in the burner. Distilled water was aspirated into the atomizer, after 30 sec, the meter reading was adjusted to 0. The meter reading was adjusted to 40 using the highest concentration standard solution (40 mg/L). Samples were thawed, mixed thoroughly and

aspirated after appropriate dilution. A calibration curve was plotted using the standard readings and the K concentration for samples was calculated using the standard curve. In house pooled breastmilk samples were used for quality control purposes. The inter-and intra assay CVs were 2.8% and 1.5% respectively.

3) Infant Gut Integrity

***Lactulose:Mannitol*:** L:M was measured by quantitative colorimetric determination using intestinal permeability kit (BioAssay Systems, US). Lactulose and mannitol were measured in separate assays using the lactulose assay kit and mannitol assay kit, respectively. The procedure was performed in a 96-well plate. BioAssay Systems lactulose assay kit is based on β-galactosidase catalyzed oxidation of lactulose, which generates D-fructose and D-galactose. The generated D-fructose reacts with specific reagent to form a colored product whose color intensity, measured at 565 nm is proportional to the lactulose concentration in the sample. BioAssay Systems, D-mannitol assay kit is based on mannitol dehydrogenase catalyzed oxidation of D-mannitol, which generates D-fructose and NADH that reduces a formazan (MTT) dye. The intensity of product color, measured at 565 nm is directly proportional to D-mannitol concentration in the sample. In-house spiked samples were used for quality control purposes with an intra-and inter assay CV of 4.2 and 2.9% respectively.

4) Child Anthropometry

Child anthropometry was measured in duplicate. Infant body weight was measured using a pediatric baby weighing scale, SECA 385 (SECA gmbh & co.: accuracy 20 gm) and length was measured using a SECA 417 (SECA gmbh & co.: accuracy 1 mm). WHO SAS macros were used to generate length-for-age, weight-for-age and weight-for-length z-scores.

5) Statistical Analysis

We used mediation path analysis to look at associations between breastmilk aflatoxin concentrations, breastmilk inflammatory markers, infant gut integrity and infant growth outcomes including length-for-age, weight-for-age and weight-for-length z-scores. All variables were continuously modelled. Breastmilk aflatoxin concentrations (AFB_1, B_2, G_1, G_2, M_1, M_2) were summed into one continuous exogenous predictor variable, used in path models.

Distributions of variables were examined, and skewed biomarker variables were log transformed, however the non-log transformed values were used for path analyses. We conducted linear and logistic regression analyses using SAS 9.4 (Carey Institute) to understand bivariate and multivariable (adjusted for maternal age, child age, sex, household wealth tertile) associations between each endogenous outcome (LAZ, WAZ, WFL) in the path models and all exogenous variables (ΣBM aflatoxins, inflammatory markers, L:M ratio). BM inflammatory markers and L:M ratio were mediators in path models. Mediation path analyses were conducted in RStudio 1.2.1335 using the Bayesian Lavaan 'blavaan' package to account for small sample sizes.

3. Results

Basic descriptives for variables used in mediation path analyses are presented in **Table 1**. Characteristics of participants in our study sample are presented in chapter 4. It is important to note that most infants in our sample had normal gut integrity, with only 3% showing L:M ratios > 0.15.

Table 1: Basic descriptives for variables in mediation path analysis (sub-sample with mycotoxin and inflammatory marker data available)

Measures	N	Mean (SD)	Median	Min	Max
Mycotoxins					
ΣBM Aflatoxins (pg/mL)	100	137.25 (164.88)	92.75	70.20	1494.80
*> *500 pg/mL MRL*	2				
ΣBM Ochratoxins (pg/mL)	100	21.50 (14.83)	11.70	11.70	89.30
Maternal Inflammatory Indicators					
IL-8 (pg/mL)	100	257.22 (291.13)	142.21	9.70	1049.22
TNF-α (pg/mL)	100	1.25 (2.42)	0.53	0	20.97
sIgA (g/L)	100	0.02 (0.012)	0.018	0.006	0.01
Lactoferrin (mg/L)	100	16.71 (7.0)	16.63	4.08	35.61
Na:K Ratio	100	0.65 (0.41)	0.62	0.10	3.35
Sub-clinical Mastitis (Na:K > 0.6)	53				
Infant Gut Integrity					
L:M Ratio	94	0.056 (0.086)	0.040	0	0.73
L:M Ratio (≥ 0.15)	3				
L:M Ratio (> 0.356 for boys)	1				
L:M Ratio (> 0.703 for girls)	1				
Infant Growth Indicators					
Length-for-age	100	-0.90 (1.18)	-0.93	-3.67	2.08
% Stunted (HAZ < -2 SD)	18				
Weight-for-age	100	-1.05 (0.91)	-1.03	-3.71	1.04
% Underweight (WAZ < -2 SD)	16				
Weight-for-length	100	-0.40 (1.09)	-0.39	-2.87	2.07
% Wasted (WLZ < -2 SD)	8				

Correlation matrices with Pearson correlation coefficients and p-values for associations between variables in path analyses are presented in **Table 2**.

Table 2: Correlation matrix[a]

Correlation Coefficient *p-value*	**[OTs]**	**IL-8**	**TNF-α**	**sIgA**	**Lactoferrin**	**Na:K**	**L:M**	**LAZ**	**WAZ**	**WFL**
[AFs]	**0.010** *0.92*	**0.051** *0.61*	**-0.035** *0.73*	**-0.046** *0.65*	**0.011** *0.91*	**-0.051** *0.62*	**-0.069** *0.51*	**-0.073** *0.47*	**0.064** *0.53*	**0.16** *0.11*
[OTs]		**0.19** *0.058*	**-0.15** *0.13*	**0.072** *0.48*	**-0.34** *0.0005*	**-0.12** *0.25*	**0.16** *0.13*	**-0.010** *0.92*	**0.046** *0.65*	**0.062** *0.54*
IL-8			**-0.035** *0.73*	**0.33** *0.0008*	**-0.16** *0.12*	**0.22** *0.027*	**-0.055** *0.60*	**0.0085** *0.93*	**0.16** *0.12*	**0.17** *0.10*
TNF-α				**-0.15** *0.13*	**-0.007** *0.94*	**-0.022** *0.83*	**-0.063** *0.55*	**-0.027** *0.79*	**-0.11** *0.27*	**-0.11** *0.26*
sIgA					**-0.059** *0.56*	**0.22** *0.030*	**0.077** *0.46*	**-0.014** *0.89*	**-0.14** *0.17*	**-0.16** *0.12*
Lactoferrin						**0.17** *0.094*	**-0.081** *0.44*	**0.021** *0.83*	**-0.066** *0.51*	**-0.10** *0.31*
Na:K							**-0.064** *0.34*	**0.020** *0.76*	**-0.11** *0.95*	**-0.029** *0.66*
L:M								**0.016** *0.81*	**-0.037** *0.58*	**-0.045** *0.51*
LAZ									**0.69** *<0.0001*	**-0.33** *<0.0001*
WAZ										**0.41** *<0.0001*

[a]Pearson-correlation coefficient and *p-value*

Results for bivariate and multivariable linear regression analyses are presented in **Table 3**, using continuous outcome variables, LAZ. We also used linear regression analysis to look at WAZ and WFL as outcomes. Only predictor variables significant in bivariate models were maternal %body fat, which showed positive associations with LAZ and WAZ (β = 0.026, 95% CI: 0.0026, 0.05, $p < 0.05$; β = 0.023, 95% CI: 0.0024, 0.044, $p < 0.05$). Na:K ratio was negatively associated with WAZ, and showed significance in bivariate analyses (β = 0.26, 95% CI: -0.56, 0.045, $p = 0.01$).

Table 3: Bivariate analyses of predictors of LAZ in univariable and multivariable linear regression models

	Unadjusted β coefficient (95% CI)	***p-value***	**Adjusted β coefficient (95% CI)**	***p-value***
Length-for-age				
BM Aflatoxins	-0.00052 (-0.0020, 0.00091)	0.47	-0.00073 (-0.0022, 0.00071)	0.32
BM Ochratoxins	-0.89 (-1.30, -0.47)	0.92	0.0046 (-0.012, 0.021)	0.58
IL-8	0.000034 (-0.00078, 0.00085)	0.93	0.00010 (-0.00071, 0.00092)	0.80
TNF-α	-0.013 (-0.11, 0.084)	0.79	-0.030 (-0.13, 0.071)	0.55
sIgA	-1.35 (-20.51, 17.82)	0.89	-0.36 (-19.51, 18.79)	0.97
Lactoferrin	-0.96 (-1.57, -0.35)	0.83	-0.0054 (-0.041, 0.030)	0.76
Na:K	0.057 (-0.32, 0.43)	0.76	0.082 (-0.29, 0.45)	0.66
Lactulose Mannitol Ratio	0.23 (-1.69, 2.15)	0.81	0.28 (-1.63, 2.18)	0.78

[1]Model adjusted for wealth tertiles of the household, child age, sex, maternal age
[*]*$p < 0.05$*

Results from logistic regression analyses using dichotomous variables for stunted (LAZ < -2) vs. not stunted (LAZ > -2) are presented in **Table 4**. We also conducted logistic regression analyses for underweight (WAZ < -2) and wasted/WFL (WFL < -2) as outcomes. An increase in maternal %body fat was associated with a higher-odds of wasting in bivariate models (OR = 1.10, 95% CI: 1.01, 1.19, $p < 0.05$).

Effect sizes for associations between breastmilk mycotoxins and length-for-age z-scores were statistically insignificant but negative in direction as would be expected, given what is

known about the role of mycotoxins in reducing infant growth. Higher levels of inflammatory markers including TNF-α, sIgA, and lactoferrin were also negatively associated with infant LAZ.

Table 4: Bivariate analyses of predictors of stunting in univariable and multivariable logistic regression models

	Unadjusted Odds Ratio (95% CI)	***p-value***	**Adjusted Odds Ratio (95% CI)**	***p-value***
Stunted (LAZ < - 2 SD)				
BM Aflatoxins	1.00 (0.997, 1.00)	0.95	0.999 (0.996, 1.00)	0.65
BM Ochratoxins	0.995 (0.96, 1.03)	0.77	1.01 (0.97, 1.05)	0.62
IL-8	1.00 (0.999, 1.00)	0.38	1.00 (0.999, 1.00)	0.31
TNF-α	1.08 (0.80, 1.46)	0.63	1.06 (0.75, 1.51)	0.75
sIgA	-		-	
Lactoferrin	1.02 (0.95, 1.10)	0.56	0.996 (0.92, 1.08)	0.92
Na:K	0.85 (0.44, 1.63)	0.62	0.91 (0.47, 1.76)	0.79
Lactulose Mannitol Ratio	0.18 (0.006, 5.31)	0.32	0.19 (0.005, 7.16)	0.45

[1]Model adjusted for wealth tertiles of the household, child age, sex, maternal age
[*]*p < 0.05*

Results from path models (IL-8 and TNF-α) are presented in **Figure 1.** Path models were generated for WAZ and WFL as outcomes, with no significant results (therefore not presented in tables and path diagrams). R^2 values describing variance in our outcome, explained by our models ranged between 0.011-0.012 for L:M, 0.027-0.028 for LAZ, 0.018-0.040 for WAZ and 0.052-0.064 for WFL as endogenous outcome.

Table 5: Direct and indirect standardized coefficients

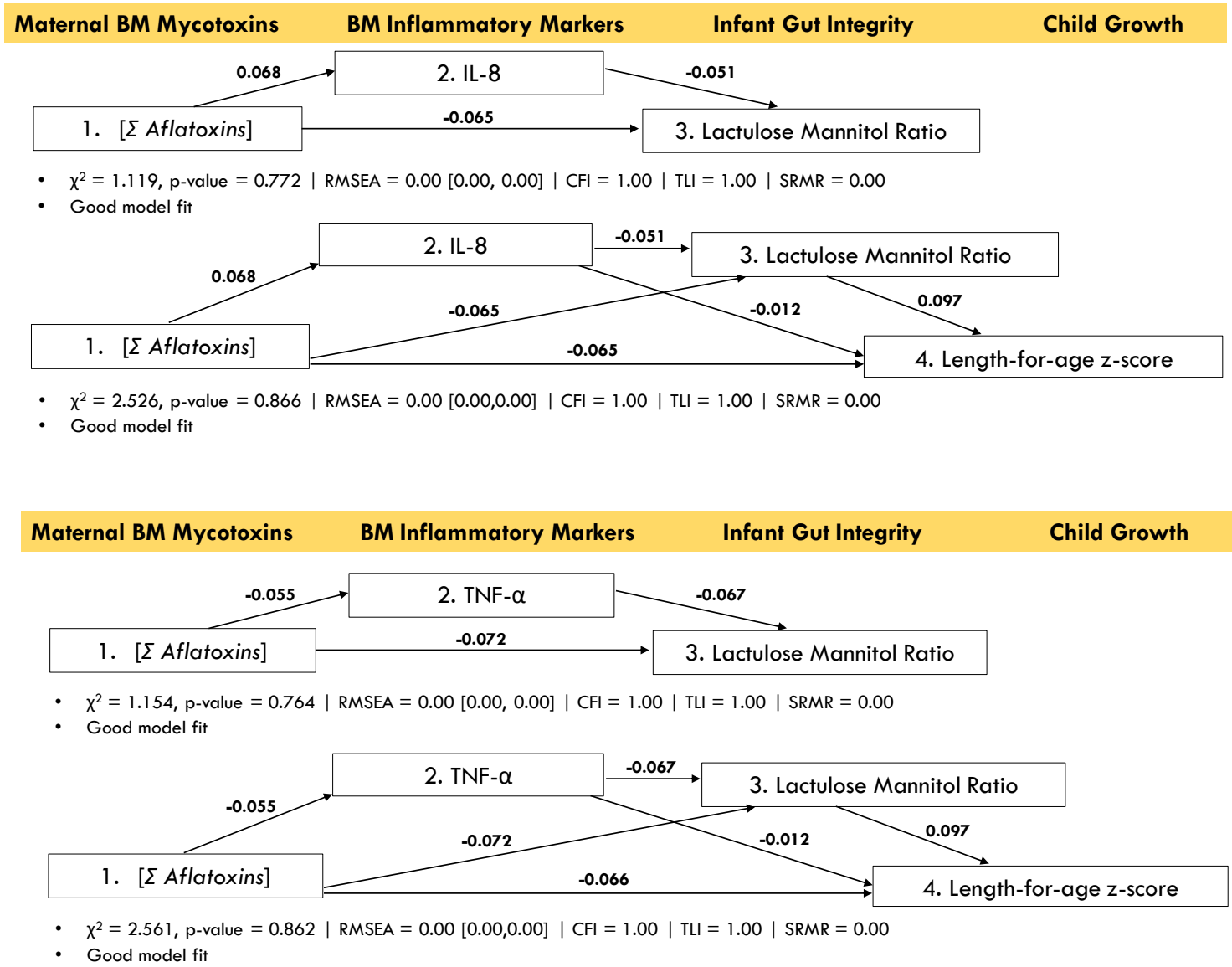
		Standardized Coefficient (SE)		
Dependent Variable	**Predictors**	**Direct Effect**	**Indirect Effects**	**Total Effects**
Lactulose: Mannitol Ratio	*[BM Aflatoxin]*			
	IL-8	-0.065 (0.00)	-0.003 (0.00)	-0.069 (0.00)
	TNF-α	-0.072 (0.00)	0.004 (0.00)	-0.069 (0.00)
	sIgA	-0.066 (0.00)	-0.003 (0.00)	-0.069 (0.00)
	Lactoferrin	-0.068 (0.00)	-0.001 (0.00)	-0.069 (0.00)
LAZ	*[BM Aflatoxin]*			
	IL-8	-0.065 (0.00)	-0.008 (0.00)	-0.072 (0.001)
	TNF-α	-0.066 (0.001)	-0.006 (0.00)	-0.072 (0.001)
	sIgA	-0.067 (0.001)	-0.005 (0.00)	-0.072 (0.001)
	Lactoferrin	-0.066 (0.001)	-0.006 (0.00)	-0.072 (0.001)
	L:M Ratio			

Table 6: Direct and indirect unstandardized coefficients

		Unstandardized Coefficient (SE)		
Dependent Variable	**Predictors**	**Direct Effect**	**Indirect Effects**	**Total Effects**
Lactulose: Mannitol Ratio	*[BM Aflatoxin]*			
	IL-8	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	TNF-α	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	sIgA	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Lactoferrin	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
LAZ	*[BM Aflatoxin]*			
	IL-8	0.00 (0.001)	0.00 (0.00)	-0.001 (0.001)
	TNF-α	0.00 (0.001)	0.00 (0.00)	-0.001 (0.001)
	sIgA	0.00 (0.001)	0.00 (0.00)	-0.001 (0.001)
	Lactoferrin	0.00 (0.001)	0.00 (0.00)	-0.001 (0.001)
	L:M Ratio			

No path coefficients were significant, likely due to a small sample size (n = 94), after accounting for missing values across variables, neither were direct or indirect mediation effects. Model fit indices including SRMR, RMSEA, χ^2, CFI and TLI all indicate good model fit across path diagrams shown in **figure 1**. Although path coefficients were insignificant and total effects were small, direction of effects were in accordance with what would be expected for associations between breastmilk mycotoxins and infant growth outcomes (negative direct and indirect associations).

Figure 1: Path models for *BM Aflatoxin → infant growth* mediated by *IL-8, TNF-α* and *L:M ratio and LAZ*



4. Discussion

A growing body of evidence suggests associations between mycotoxin exposure and impaired gut integrity in animal and cell line studies (Mapesa et al., 2016; Owino et al., 2017). Our exploratory research applied mediation path analyses to understand associations between breastmilk mycotoxins and infant linear growth. While we were not powered to detect statistically significant associations, we did find direct and indirect effect sizes to be in the expected directions, suggesting a need for further investigation using larger sample sizes and data from children over a larger age range. A minimum sample size of 150 is needed to conduct mediation path analyses, as per Schoemann et al., (2017).

In animal models and in *in vitro* cell studies, mycotoxins have been shown to result in alterations to biological structure and function in various tissues and systems, including the intestine, liver and kidney epithelia, with additional impacts on the nervous, reproductive and immune systems (Bondy & Pestka, 2000; Campbell et al., 2004; Oswald et al., 2005). Correlations have also been found for the associations between aflatoxin exposure and gut health (Jolly et al., 2007). In animal models, particularly chickens, small intestines of those exposed to AFs have been seen to weigh less than the intestines of unexposed chickens, suggesting decreased absorptive capacity (Yunus et al., 2011). Chickens exposed to AFs have also been seen to have a reduced crypt depth but not villus length, with an increased villus: crypt ratio, suggesting reduced intestinal absorptive capacity. These physical alterations may in turn be associated with zinc deficiency. AFs also affect gut health and growth by inhibiting protein synthesis, but not apoptosis of intestinal cells directly due to exposure.

The effects of fumonisin exposure in animal models such as pigs have been studied and suggest that exposure in porcine epithelial cells can lead to decreased transepithelial electric

resistance (indicating increased epithelial permeability). Further, FUM-exposure has also been shown to downregulate local IL-8 measured in the intestine, indicating that exposure affects mucosal immunity and can increase risk of enteric infections. Disruptions in sphingolipid metabolism and an increased risk of infection due to altered mucosal immunity are likely caused by fumonisin exposure (Smith et al., 2012).

Mycotoxins including ochratoxins (OTA), deoxynivalenol and patulin have been shown *in vivo* to have enteropathogenic activities including altering intestinal function, leading to malabsorption, malnutrition, diarrhea, vomiting and intestinal inflammation (Hunder et al., 1991). Using Caco-2, T84 and HT-29 cells, DON and OTA have been shown to compromise the intestinal barrier function by altering tight junction complexes (Maresca et al., 2001, 2002; Sergent et al., 2006); they have also been shown to inhibit intestinal nutrient absorption, particularly sodium-dependent absorption of D-glucose (Maresca et al., 2001, 2002).

DON and OTA can cause inflammatory activity which can result from a direct stimulatory effect on the production of pro-inflammatory cytokines by intestinal epithelial cells (IECs). DON and OTA have been shown to directly stimulate cytokine production by immune cells (Campbell et al., 2004). These mycotoxins may therefore have an effect on cytokines and interleukin production by gut epithelial cells. Production and secretion of proinflammatory cytokine, IL-8 has been reported in Caco-2 and Int407 cells, in response to DON (Moon et al., 2007). DON is also known to potentiate effects of pro-inflammatory stimuli including lipopolysaccharides (Islam & Pestka, 2006) and bacteria (Mbandi & Pestka, 2006) on immune cells. Mycotoxins may also cause indirect inflammation in the intestine by opening of tight junctions, which allow for entry of luminal antigens and bacteria, normally restricted to the gut lumen by the intestinal barrier function, leading to tissue inflammation and invasion of

commensal and pathogenic bacteria (Maresca et al., 2008; Clark et al., 2005). Maresca et al., (2008), demonstrated that DON directly stimulates production of IL-8 secretion from human intestinal epithelial cells (Maresca et al., 2008; Instanes & Hetland, 2004). OTA induced oxidative stress can also alter intestinal permeability, which has been found to be associated with apoptosis in the intestinal IPEC-J2 cells (Anderson et al., 2016; Wang et al., 2017). Inflammatory pathways are also affected by OTA, namely expression of inflammatory cytokines, such as IL-8, which are decreased in the intestines of piglets exposed to the toxin (Marin et al., 2017). We did not conduct advanced mediation analyses on associations between ochratoxins in breastmilk and infant growth. Preliminary Pearson correlation coefficients in correlation matrices for associations between OTs, IL-8 and lactoferrin suggest significant associations. Future studies should explore these relationships in more detail.

Few studies have examined the effects of AFM_1 and OTA cytotoxicity in human intestinal Caco-2 cells (Gao et al., 2018). As multiple exposures are likely the most common in biological matrices such as milk, the simultaneous presence of many mycotoxins in food may lead to interactive effects such as additive, synergistic and antagonistic (Ficheux et al., 2012). Gao et al., (2018) have shown synergistic effects on intestinal integrity of Caco-2 cells *in vitro*. Results from this analysis show that AFM_1 + OTA exert synergistic effects on intestinal epithelial integrity at 4 µg/mL. This synergistic effect may be associated with down-regulation of four pathways related to intestinal integrity in cells treated with AFM_1 + OTA, namely TNF-α signalling pathway, regulation of actin cytoskeleton pathways, focal adhesion and adherens junctions; down-regulation of proteins associated with gap junction, inflammatory mediator regulation of TRP channels and calcium signalling pathways were also implicated. Additionally, these authors note that multiple mycotoxin exposure also leads to activation of immunity-related

pathways that enhance epithelial immune responses. As co-contamination of mycotoxins is common, it is prudent to examine their presence and interacting effects in biological matrices such as serum, urine and breastmilk. Exposure to multiple mycotoxins in biological systems is likely the norm (Schatzmary & Streit, 2013).

Although no studies have examined associations between mycotoxins in breastmilk and inflammatory markers, studies conducted in dairy animals have shown associations between mycotoxins and sub-clinical mastitis ($0.6 < Na:K < 1.0$ indicating SCM; $Na:K > 1.0$ indicating mastitis), resulting in a decrease in milk production (Fink-Gremmels, 2008; Brown et al., 1981) . Mastitis, or inflammation of the mammary tissue is known to increase concentrations of immune factors such as IL-8, an inflammatory cytokine in breastmilk of humans, leading to sub-optimal growth in infants (Filteau et al., 1999; Filteau et al., 1999; Willumsen et al., 2003; Gomo et al., 2003).

With regards to biological plausibility of pathways, based on preliminary animal studies and *in vivo* cell line studies, it is known that mycotoxins can impair gut integrity by impacting tight junctions and intestinal barrier function. Studies on breastmilk as a source of exposure to these mycotoxins are also growing in number, with our results presented in chapter 3 adding to the evidence base in the Indian context. Our findings add credence to animal and cell line studies and represent among the first to explore these associations from an epidemiological perspective. Results are in line with hypothesized associations between breastmilk aflatoxins and their role in impairing infant growth. Negative direct, indirect and total effects in path models, in our preliminary exploratory analyses suggest that trends and directionality of associations between aflatoxins and infant growth are in line with our hypotheses. However, the mediating role of breastmilk inflammatory markers and infant gut integrity warrant further investigation.

Given our overall findings, it is prudent to further understand the impact of breastmilk mycotoxins on sub-clinical and clinical mastitis, and infant gut integrity leading to growth faltering. Longitudinal studies with sample sizes > 300 should ideally be conducted among children living in low income communities, where risk of exposures is the greatest (LeRoy et al., 2015). Major limitations of our analyses include power and sample size, which do not allow us to draw conclusions. Our analysis was restricted to aflatoxins as other mycotoxins were not detected at biologically relevant levels to merit further analysis.

As infants in our sample were only between 2-4 months of age, it is unlikely that we would observe adverse effects on the gut leading to compromised integrity, even among those who are exclusively breastfed, due to relatively short duration of cumulative exposure. Only 2 children in our sample were exposed to ΣAFs in BM > than MRL levels for the mycotoxin. As noted gut integrity was not impaired in > 96% of children and only 18% were stunted, 16% underweight and 8% wasted, adding further plausibility to our findings of a lack of associations, due to a lack of variation in predictor variables, despite them being modelled continuously.

Additionally, we were not powered to detect associations with our limited sample of women for whom mycotoxin and breastmilk inflammatory biomarkers were available. Longitudinal studies with larger sample sizes and samples from across the lactation period are needed to understand fluctuations in levels of mycotoxins and account for variations in human milk composition, which is known to be dynamic (Ballard & Morrow, 2013).

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Chapter 7: Discussion

7.1 Summary of Key Findings

There is a high prevalence of early growth faltering in the first 6 months of life in India and many questions remain on potential key drivers. My dissertation focuses on one overlooked area, the role of mycotoxin and pesticide exposure among breastfed infants. While our ultimate long-term objective is to understand relationships between environmental exposures and child growth, we first needed to develop methods to quantify environmental toxins in milk samples, understand exposure in breastmilk and food samples and quantify potential risk to lactating mothers and infants in the area. Below the main findings from the four aims of this dissertation are as follows:

Aim 1: *Quantification of aflatoxin and ochratoxin contamination in animal milk using LC-MS/MS: a small-scale study*

We developed and validated an ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) selected reaction monitoring (SRM) method to detect 6 Aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2) and 2 Ochratoxins (A & B) in n = 38 cow, goat, buffalo and pasteurized commercial milk samples from southern India. Overall method performance was in accordance with bioanalytical guidelines set by the USFDA. We applied our validated method to local animal milk samples and noted mean concentrations of AFB_2: 38 pg/mL for in goat milk, AFM_1: 331 pg/mL, 406 pg/mL, 164 pg/mL in cow, goat and pasteurized milk, respectively. We also found AFM_2 at 249 pg/mL, 375 pg/mL and 81 pg/mL in cow, goat and pasteurized milk samples, respectively. Additionally, 90% of cow, goat and pasteurized milk samples were above European Union (EU) limits of 50 pg/mL (0.05 µg/kg) for AFM_1 and 40% of goat milk samples were above the Food Safety Standards Authority of India (FSSAI) limit of 500 pg/mL (0.5 µg/kg). The low dynamic ranges for the method in addition to a need for only 1 mL of matrix

make this method ideal for biomonitoring for mycotoxins in animal and human breastmilk samples. AFM_1 was found in a small sample of animal milk samples at levels above regulatory limits, thus warranting further attention in larger sample sizes of dairy milk and animal feed.

Aim 2: *Risk of dietary and breastmilk exposure to mycotoxins among lactating women and infants 2-4 months in Northern India*

Method developed in aim 1 was used to quantify 6 AFs and 2 OTs in n = 100 human breastmilk and n = 30 animal milk samples from Haryana, India. AFB_1, FB_1, and DON were quantified in n = 298 assorted food items. We quantified AFM_1 in 41% of human milk samples and 93% of animal milk samples. AFB_1 and FB_1 were detected in several food items at > 30 µg/kg, the maximum limits set by FSSAI. DON was not detected. Mothers and infants were above PMTDI limits set for AFM_1 due to milk consumption. Maternal intake of AFB_1 via consumption of rice and flour were also above the 1 ng/kg bw/day PMTDI limits. AFB_1 and AFM_1 were found in cereal grains, animal milk samples and breastmilk samples in our community food system. Further studies are needed with larger samples sizes to validate these findings and develop interventions to address mycotoxin exposures, to mitigate risks to mothers and infants.

Aim 3: *A mixed-methods study of pesticide exposures in breastmilk and community and lactating women's perspectives from Haryana, India*

We quantified organochlorine (and tested for organophosphate and pyrethroid) pesticides in n = 75 human breastmilk samples from Haryana, India. We found DDT (< LOQ – 28 µg/L) and DDE (< LOQ – 107 µg/L) in 4% and 5% of samples, respectively. We conducted qualitative FGDs (n = 9) and IDIs (n = 30) with community members and lactating women to understand perceptions of and knowledge, attitudes and practices related to pesticide use. Due to gendered

engagement in farming, lactating women in study communities do not directly handle pesticides, thus precluding direct inhalation and ingestion routes and occupational pathways of exposures. Although pesticide exposures in breastmilk are not of public health risk, further research to understand pesticide exposures in the food system in these contexts, and in larger sample sizes of breastmilk, with improved method performance (< LODs and LOQs).

Aim 4: *Mediation path analyses of associations between breastmilk mycotoxins and infant growth outcomes*

We examined associations between cumulative breastmilk aflatoxin exposure in n = 100 breastmilk samples, breastmilk inflammatory markers (IL-8, TNF-α, sIgA and lactoferrin), lactulose-mannitol ratio in infant urine and infant length-for-age, weight-for-age and weight-for-length z-scores using mediation path analysis. This was a learning exercise and we were not powered or designed to detect associations. As expected, we did not detect significant associations in our regression analyses or mediation models. There are many reasons for null findings: small sample size, child age (2-4 mo. captured), and infants in our sample have healthy guts based on L:M ration (only 3% with L:M ≥ 0.15). This analysis was valuable to gain analytic skills and understand how to apply this methodology to future projects.

Findings from this dissertation add to a growing body of global evidence showing moderate to high levels of mycotoxin contamination in animal and human milk samples (Cherkani-Hassani et al., 2016; Coppa et al., 2019; Fakhri et al., 2019). Recent systematic reviews of the literature on breastmilk mycotoxin exposure have reported between 73 studies published between 1984 – 2019 from countries in Africa, Americas, Asia and Europe (Cherkani-Hassani et al., 2016; Coppa et al., 2019). Very high levels of mycotoxins were reported in Egypt (200-19,000 ng/L, n = 150), Sudan (7-2561 ng/L, n = 94), Iraq (100-3010 ng/L, n = 20) using

methods including ELISA, HPLC/FD and TLC, respectively (Tomerak et al., 2011; Elzupir et al., 2012; Qadir & Ali, 2014). Our results, for AFM_1 (3.9-1200 ng/L) in breastmilk, although lower, utilize a method that is significantly more sensitive and specific, thus improving validity of quantitation and overall confidence in our findings. In relation to FSSAI/USFDA (500 pg/mL) and EU/Codex (50 pg/mL) regulatory limits for AFM_1 in milk samples, only 1% of our study samples exceeded FSSAI limits and 4% exceeded, the more conservative EU limits for AFM_1 in milk and 11% exceeded the EU limit for AFM_1 in infant foods (25 pg/mL).

Heterogeneity in geographical locations (tropical and temperate climates), season of collection (dry versus wet), socioeconomic status and maternal dietary patterns (monotonous maize or cereal based diets vs. improved dietary diversity), in addition to methods for exposure assessment, make it challenging to draw comparisons between the results of studies (Polychronaki et al., 2006, 2007; Galvano et al., 2008; Magoha et al., 2014; Ortiz et al., 2018). This is the first study to quantify mycotoxins in human breastmilk from India, and amongst only a few to have examined this issue in South Asia (Khan et al., 2018). In addition, ours is the first to use UHPLC-MS/MS for quantification, when compared to other studies from the region. Prior studies have used less sensitive and specific methods such as ELISA. Khan et al., (2018), in their study from Pakistan, found 75% (n = 125) of breastmilk samples tested, to be positive for Aflatoxin M_1 with limit of detection at 1 ng/L and LOQ of 3 ng/L. Our LOD for AFM_1 was 7.8 ng/L in comparison. These authors noted that 6.4% of samples were over EU permissible limit (25 pg/mL), with mean prevalence ranging between 3(±0.8)-17(±0.2) pg/mL. Women from the lowest socio-economic classes had higher prevalence (poor → 28±0.5 pg/mL > middle class → 21±0.4 pg/mL > rich → 18±0.4 pg/mL). We did not see significant associations with SES variables in our analyses, as reported in other contexts (Yard et al., 2013; LeRoy et al., 2015).

Authors of the research conducted in Pakistan, note that high levels of AFM_1 in mother's milk from this context may be explained by lower literacy rates, suitable weather for production of AFs, higher consumption of foods that are vulnerable to AF contamination and weak implementation of regulations to control limits of mycotoxins in the food supply chain (Khan et al., 2018; Ismail et al., 2016).

Our findings for mycotoxins in animal milk samples are in concordance with a nationally representative study on dairy milk for human consumption conducted by the FSSAI in India, which found 5% of over n = 6000 samples to be contaminated with AFM_1 (FSSAI Milk Survey, 2018). We found AFM_1 in 100% of buffalo and pasteurized commercial milk samples (n = 30), collected across summer, monsoon and fall. Rastogi et al., (2004), found AFM_1 in liquid milk from Lucknow, at between 28-164 ng/L and in infant milk products at 65-1012 ng/L. Although we did not find precursor, AFB_1 in infant formula, AFM_1 levels in our liquid milk samples ranged between 3.9-4158 ng/L. Our findings for AFB_1 and FB_1 in food commodities are also in line with other studies that have reported 44-75 % of maize and groundnuts to have detectable levels of AFB_1 and 84% and 91% of maize and pearl millet samples to be contaminated with FB_1 in Uttar Pradesh, North India (Wenndt et al., 2020 unpublished results).

Our results for analysis of pesticides in human milk samples are in contrast to other studies that have found moderate to high levels of pesticides in Indian breastmilk (Devanathan et al., 2009; Bedi et al., 2013; Bawa et al., 2018). As discussed extensively in Chapter 5, our LODs and LOQs are higher than those reported in the literature. Thus, while we were unable to detect trace concentrations of OCs, OPs and pyrethroids in our samples, the method is able to detect levels of public health importance (below MRLs). We found 1.3% of breastmilk samples to have levels of p,p'-DDT and p,p'-DDE over MRLs for milk. We reviewed several recent (2006-2018)

papers that have presented analyses of breastmilk pesticides in India (presented in chapter 2). OCs, OPs, pyrethroids and carbamate pesticides have been detected in human milk from several north India cities (Delhi, Agra) and states (Rajasthan, Assam, West Bengal, Punjab, Himachal Pradesh). DDTs, specifically, have been detected at levels ranging between 0.001365-5062.5 µg/kg (we found DDT at <LOQ-28 µg/L and DDE at < LOQ -107 µg/L). Our preliminary screening study suggests that breastmilk pesticide concentrations do not pose a public health problem in this population. However, this does not preclude their presence in breastmilk or the potential for infant exposure, further upstream, from dietary sources. Our qualitative findings add further credence to breastmilk pesticide values, and showed that all activities associated with the use of farm chemicals are conducted away from the household. Community members recognize the need for pesticide use to ensure crop yields, despite differing views of these chemicals as medicines and poison. As farming is gendered in this context, women in many households do not engage in this activity. Pesticide handling and use remains the purview of male members of the household, thus limiting risk of direct exposure to lactating women.

We conducted a preliminary method learning exercise to understand novel associations between environmental exposures in breastmilk samples, inflammatory markers in breastmilk and infant gut integrity. Reverse causality and residual confounding cannot be negated in a cross-sectional observational design (Sattar et al., 2017). Overall null results for epidemiological associations in our analyses are in alignment with what would be expected given our sample size, age of infants enrolled in the study and lack of variation in infant gut integrity and infant growth outcomes. Although epidemiological associations in this small study were not significant, mycotoxins and pesticides were detected in breastmilk and the food system (we did not have food systems data for pesticides, although others have reported high levels in the north Indian

context). Our findings, therefore suggest a need for more comprehensive, longitudinal investigations with children in older age groups (> 6 months and up) to better understand the impact of chronic and longer-term insults related to environmental exposures during lactation and beyond.

7.2 Strengths & Limitations

Strengths

7.2.1 Study design, sample size and sample collection

Study participants were recruited from an existing pregnancy surveillance database maintained by our partner organization in New Delhi, India. Overall refusal rates (n = 3 refusals), for this study were low, as was participant attrition (n = 2 respondents left study area), likely driven by positive rapport and long-term engagement of households with implementing partner organization.

An important strength and *design* component of this cross-sectional observational study is our ability to capture seasonality. Women were enrolled in the parent study over the course of a year, to capture seasonal effects. We drew a stratified random sub-sample from the larger study to capture these seasonal effects in our secondary analyses for mycotoxins and pesticides, thus ensuring 12-month representation. On a similar vein, we also captured seasonality (summer, monsoon, fall) for food samples collected for mycotoxin analysis (described in chapter 4). Our ability to capture various levels of the community food supply chain, i.e. village (n = 11 communities, 20 shops), retail (n = 6 mid-level markets, 15 shops) and wholesale (n = 3 regional markets, 9 shops) was an added nuance. Although no statistically significant differences were observed for location of procurement of food samples, we did note seasonal variations in levels

of mycotoxins in several food items. Few studies have examined seasonal variations in concentrations of breastmilk mycotoxins and pesticides (El-Nezami et al., 1995; Lamplugh et al., 1988; Maxwell et al., 1989). These authors found frequency of detection of aflatoxins in human breastmilk to be higher in the wet season compared to dry season. More recently, Skaug et al., (2001), found OTA to be higher in winter. Polychronaki et al., (2007), found high levels of AFM_1 in breastmilk during summer months, namely April.

Despite small *sample sizes* for this exposure assessment, ours (n = 100 for mycotoxins and n = 75 for pesticides) are in line with other studies that have examined such exposures in breastmilk samples. Sample sizes for breastmilk mycotoxin studies typically vary between 40 to > 200 (Anthony et al., 2016; Andrade et al., 2013). For studies of pesticides in breastmilk, sample sizes range between 10 to >500 (Behrooz et al., 2009; Raab et al., 2013).

It is important to note that collection of biological samples, particularly in non-clinical field-based settings is challenging for several reasons. These include consent and compliance from study participants, challenges associated with concurrent infant feeding and maintenance of schedules to correspond with data collection activities, general logistics and cold chain management including storage (-2ºC to -80ºC) of samples to ensure their integrity and avoid degradation (National Academy of Science/Measure Evaluation, 2000). Additionally, analyses of biomarkers and associated sample sizes are limited by factors such as time, expertise, availability of specialized equipment and cost.

The challenges notwithstanding, best practices were put in place for breastmilk sample collection, including at point of procurement and during transport to and between laboratories. A 14-day household contact period necessitated by the deuterium oxide dose to mother protocol to assess breastmilk volume and maternal body composition, and collection of infant urine for

lactulose-mannitol testing, allowed for extended contact with women and children enrolled in the study. This also allowed field teams to administer survey components on different days, to reduce respondent burden. Recall bias may be an issue for certain questions posed in the survey i.e. infant birth weight, among others, as these data were only verified with documented records such as hospital discharge cards/birth certificates/child growth cards, where they were readily available. Our implementing partner in New Delhi has several decades of experience with running large scale field trials employing collection of biological data from respondents. The availability of trained phlebotomists and laboratory technicians, in addition to experienced enumerators and coordinators, ensured high quality data collection, and adherence to strict protocols involved with cold chain management and shipping to partner laboratories in southern India, for sample analysis.

7.2.2 Methods and data breadth

The findings presented in this dissertation are part of a larger National Institutes of Health (NIH) R21 study meant to examine maternal nutrition and lactation performance in Faridabad district, Haryana. Maternal breastmilk samples were collected and archived as part of this work, and in turn used to perform several secondary analyses presented in this dissertation.

The availability of **biological samples** is a great strength of the work presented here. Our ability to detect and quantify biomarkers of exposure provides confidence in our findings, beyond mere hypothesization. A strength of our study is availability of rich data on other physiological measures including: 1) breastmilk volume and intake; 2) inflammatory markers in breastmilk; 3) infant gut integrity measures in urine and; 4) maternal and infant anthropometry.

In addition to biological sample data, we also collected **survey data** from n = 100 households (sub-sample of n = 232) to examine potential confounders which can include

demographic and socioeconomic factors, *household food security*, *environmental and food systems exposures*, *infant and young child feeding practices*, and *maternal dietary intake (24-hour recall and food frequency questionnaire).* Availability of these data allowed us to examine and control for key confounders including household SES and maternal age, in TOBIT regression analyses. Additionally, availability of dietary data also allowed us to examine risk and conduct exposure assessments for aflatoxins, in mothers and infants in relation to actual reported quantities of food consumed per kg body weight per day.

Third, we also collected data using **qualitative methods** including in-depth interviews and focus group discussions with lactating women and community members, respectively, with the aim to understand knowledge, attitudes, practices and perceptions associated with the household food chain. Knowledge of the context in addition to the first author's ability to converse with respondents directly in Hindi, and conduct all field work, as opposed to need for a third party, or translator was an important benefit. Additionally, use of verbatim transcripts and assessment of inter-coder reliability are also important strengths of our qualitative work.

The objective of this component was to broaden the scope of our research question to include varied perspectives by engaging key community members including mothers and fathers-in law and frontline workers (FLWs). Concordance of findings from qualitative and biomarker findings, allows for a holistic understanding of the situation pertaining to pesticide exposure in our context. Despite the pilot nature of this study, our mixed-methods approach strengthens preliminary findings, while speaking to a need for additional research to understand the extent of mycotoxin and pesticide exposures in study communities, and associated impacts on maternal and child health.

7.2.3 Analytical and statistical methods

It is important to note that risk of dietary exposures, such as those examined here can be assessed using FFQ data and 24-hour recalls, however these are prone to measurement error, which attenuate estimates of disease related risks and reduce statistical power to detect significance, thus obscuring relationship between diet and disease (Kipnis et al., 2003). To mitigate issues associated with dietary recalls in assessment of exposures, we examined biomarkers of exposure (and not mere bio-measures), in our study which are known to be correlated with dietary intake of parent xenobiotics. This adds further credence to the validity of our findings that these exposures are an issue within the food system and warrant further investigation (Turner et al., 2012).

In line with the point above, the added availability of high-quality dietary data acquired using a context-specific and validated food-frequency questionnaire to assess dietary determinants of maternal mycotoxin exposure in breastmilk samples, was a strength (Bowen et al., 2012). In addition, availability of 24-hour recall data for assessment of food quantity intake enabled our exposure assessment.

The highly sensitive and specific nature of analytical methods used in this study, reduce the risk of measurement error/bias. A gold-standard method, namely ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) was used to detect and quantify mycotoxin concentrations in breastmilk samples. Compared to other methods such as high-performance liquid chromatography coupled to fluorescence detectors (HPLC-FD), NMR spectroscopy, enzyme-linked immunosorbent assays (ELISA) among others, UHPLC-MS/MS is more sensitive and specific, allowing for targeted analysis and detection of trace quantities of analytes in complex biological matrices such as milk (Rathod et al., 2019). The use of deuterium

labelled internal standards (ISTDs) is another important strength of our analysis, these helps mitigate matrix effects caused by composition of complex matrices such as milk (De Nicolo et al., 2017). An important caveat is the use of only 1 set of ISTDs for each group of mycotoxins analyzed, namely AFB1-D_3 and OTA-D_5, in our analysis. Although corresponding ISTDs for each analyte measured in our analysis is ideal, cost constraints coupled with availability of deuterated STDs prohibited us from acquiring these, therefore limiting our protocol to use of the aforementioned compounds. Our ability to use a small volume of milk (1 mL) and straightforward extraction protocol (no use of solid-phase extraction, derivatization etc) allowed us to assay upwards of 30 samples per day. This validated method, with its relative time efficient protocol comes with cost benefits (estimated cost per sample between INR 3000-5000 or USD 40-70) and can be used for larger scale analyses of human and animal milk samples.

Another major strength of this work is availability of data on breastmilk volume consumed by infants using the deuterium oxide 'dose-to-mother' technique, which also provides information on total body water in mother and her body composition (IAEA, 2010). The traditional method for measurement of breastmilk intake is to "test weigh" infants before and after a feed. This method is time consuming and can disturb normal feeding patterns of participants (Limon-Miro et al., 2017). Given availability of accurate information on breastmilk volume consumed, we were able to generate 'calculated daily intake' (CDI) values for mycotoxins consumed by infants, in contrast to 'estimated daily intake' values which as the term suggests are estimates. The availability of these data, further strengthens the findings of our risk assessment for infant mycotoxin exposure, described in chapter 4.

Our analysis of maternal determinants of mycotoxin exposure in breastmilk is unique in its use of TOBIT regression for censored data. Prior studies that have examined determinants

have used linear and logistic regression or ANOVA for non-parametric censored data (Polychronaki et al., 2007; Galvano et al., 2008; Bogalho et al., 2018). TOBIT or censored regression models are used to estimate linear relationships between variables when dependent variables exhibit left- or right-censoring (UCLA Statistics, 2020). As discussed in chapter 4, mycotoxin values in breastmilk were < LOD (left-censored) for between 57-92% of samples in our study. Censoring from below (our case), occurs when cases with a value at or below a threshold (LOD), all take on the value of that threshold (1/2 LOD, applying medium bound scenario), so the true value for contamination in samples may be equal to the threshold or lower. The use of TOBIT regression yields unbiased coefficient estimates, as compared to the use of linear regression for censored data (Sims et al., 2012; Wang et al., 2016).

Limitations

7.2.4 Selection bias and generalizability

Given that women were recruited into this study based on their ongoing engagement with the implementing organization, selection bias is a valid concern. Our partner organization recruited women with children 2-4 months of age, who were breastfeeding, met other study inclusion criteria and were part of an existing pregnancy surveillance database set up as part of prior studies, run by the organization (other inclusion/exclusion criteria described in detail in chapter 5, including length of stay at residence, current breastfeeding status, use of tobacco products etc).

It is possible that eligible women not on the existing database were not recruited, and women from the most vulnerable households within communities not sampled, thus implicating potential confounding by socioeconomic status. The aforementioned factors also relate to generalizability of our findings to the rest of Haryana and northern India, more broadly. Women in our sample were generally healthier (in NHFS-4, 18.2% of Haryanvi women had BMI < 18.5 kg/m^2 vs. 11%

in our sample; 64% of women were anemic (Hb < 12 g/dl) in NFHS-4, compared to 61% in our samples). Additionally, in our overall sample, 36% of women had > 10 years of education, compared to 39.7% in rural Haryana, as per NFHS-4.

Prior studies have documented significant associations between maternal region of residence and OTA concentrations in breastmilk (Skaug et al., 1998), and associations between mycotoxins in breastmilk and residing in villages (Dehghan et al., 2014; LeRoy et al., 2015). It is likely that women who are the most vulnerable to adverse environmental and dietary exposures within selected study communities, were not captured in our sample. Future studies should consider more representative sampling.

Given the particular demographic and geographic location of study communities, close to Faridabad city and New Delhi, ease of sample transport to a laboratory for cold storage was ensured. This also meant that we did not capture communities in very rural parts of Haryana, with lack access to urban centers and associated amenities. The issue of confounding by community SES is important to consider. SES at the household and community level, is an important proxy for other maternal determinants such as diet quality (poor grain quality) and dietary exposure, women's status and education, engagement in farming and occupational exposure to mycotoxins and pesticides (Bardia et al., 2004).

The results from our work may therefore not be representative of the full extent of exposure in the north Indian state of Haryana. Women from rural agrarian communities, internal migrant households (from other rural parts of north India) and those living in extreme poverty may in fact experience higher levels of exposure, than those documented in our work (LeRoy et al., 2015). Future studies should consider sampling populations living in remote and rural regions of northern India, with a particular focus on households of the lowest socioeconomic classes and

most marginalized castes. Across northern India, farm workers who engage in *"battaiya"* or daily wage farm labor including pesticide spraying, come from scheduled castes and tribes and from the most economically marginalized backgrounds. It is essential to capture these vulnerable populations, as the true extent of exposure and related consequences are likely to be felt most adversely by these groups.

7.2.5 Study design and biomarkers

As this study was a proof of concept pilot, we were limited to a cross-sectional observational design to generate preliminary findings on our research question and exposures of interest. This design precludes us from drawing causal conclusions about the impact of mycotoxins and pesticides on infant gut integrity and growth outcomes, limiting our conclusions to associations between exposure and outcomes, at best. An important limiting factor was also the age of children enrolled in the study. At between 2-4 months of age, exposure to breastmilk toxicants is relatively short-term. Additional insults to the infant gut are also temporally limited. We noted this in terms of our findings regarding infant gut integrity, where lactulose mannitol ratios were normal, except for 3 children (L:M $\geq$ 0.15) in our sample. In a systematic review of the literature where L:M ratios have been used to evaluate environmental enteric dysfunction in children, Denno et al., (2014) found that mean L:M values from Asia and Africa were > 0.12, the reference mean for healthy children 3-15 months of age, in the UK. In our study, 5 children had L:M ratios > 0.12. Campbell et al., (2003), in a cohort of Gambian children between 8 weeks and 64 weeks of age, found that L:M ratios more than doubled between the ages of 3-12 months (correlation between age and L:M, $r = 0.44$, $p < 0.001$). This is due to increased lactulose ($r = 0.18$, $p < 0.001$) and decreasing mannitol ($r = -0.14$, $p < 0.001$) excretion with age, reflecting an age-related increase in intestinal permeability (Campbell et al., 2003). In this study, intestinal

permeability accounted for 22% of growth failure, with progressive increase in prevalence and severity of enteropathy after 3 months of age, a time when weaning foods are likely introduced, resulting in onset of growth failure (Campbell et al., 2003.

It is important to note that at the developmental stage (2-4 mo.) captured in our sample, most growth faltering in infants may be driven by intra-uterine growth restriction, as opposed to environmental or nutritional insults and deficiencies (de Onis, 2017). Unfortunately, it was beyond the scope of our study to examine prenatal exposures. As discussed in chapter 2, L:M ratio is a crude urinary biomarker for infant gut integrity, and although urine is easier to acquire in field settings, stool biomarkers of gut integrity such as neopterin (NEO), myeloperoxidase (MPO), and alpha-1-antitrypsin (AAT) are more reliable (Kosek & MAL-ED Network Investigators, 2017).

Breastmilk was also only collected from women once during the course of lactation, at one point in the day by manual or pump expression of one full breast. Ideally, we would want to collect breastmilk samples at various points in the lactation period, but also sample breastmilk at multiple points during a day to assess diurnal variations in excretion of exposures from fat tissue in the breast into milk, and potential differences between feeds (Wu et al., 2018).

In addition, we did not analyze maternal breastmilk biomarkers for FB_1 and DON. Several other mycotoxins have also been detected in breastmilk samples from across the world (Valitutti et al., 2018; Memis et al., 2020; Ezekiel et al., 2020). Future studies should assess the presence of FB1, DON, beauverucin, enniatins B, zearalenone and other mycotoxins in human milk samples.

Sampling limitations for food items include purchase of items as provided by vendors. Ideally one would want to conduct some form of lot quality assurance and use a standard

sampling spear to capture variation in grain quality within lots (sacks) (Wenndt et al., 2020). This is not always possible to do in a field-based setting. We instead asked vendors to provide us with samples as would be purchased by households for regular use. Future, systematic sampling of grain products should follow standard protocols.

As discussed in chapter 5, our LODs and LOQs for pesticide detection in breastmilk samples were high. Although a comprehensive list of 53 OCs, OPs and pyrethroids was assessed, it would be prudent to also examine carbamate pesticide residues in Indian breastmilk samples, due to their extensive use in agriculture and vector control programs (Abhilash & Singh, 2009).

7.2.6 Sample size and power

Despite our sample sizes for exposure assessment being characteristic of other studies to have conducted biomarker analyses, we were not powered to detect associations in our mediation path analyses with infant growth measures as our outcomes. It is estimated that a minimum sample size of 150 is needed to attain 80% statistical power to detect indirect effects in mediation models (Schoemann, Boulton & Short, 2017).

Larger sample sizes are also needed for food sample mycotoxin assessments. An important limitation was our inability to assess pesticide exposures in the food supply in study communities. Due to budgetary limitations, we were unable to understand whether direct ingestion and dietary exposures to pesticides are a concern, and put upstream food systems context to our breastmilk pesticide findings. Additionally, we were unable to collect food samples from households where breastmilk was sampled, and had to extrapolate food sample exposures to the community and market levels. Sampling of food items from individual households, would have allowed us to more reliably contextualize breastmilk mycotoxin findings. This is another important limitation. Ideally, a total diet study or collection of food

samples from plate ready meals would allow us to sample and test raw and cooked food items directly consumed by individual women at between 3-4 time points in a year, accounting for seasonal variations (Carballo et al., 2019).

7.2.7 Analytical limitations

An important analytical limitation was the use of enzyme-linked immunosorbent assays to screen for and quantify AFB_1, FB_1 and DON in food items. LC-MS/MS and GC-MS based approaches are preferable for food matrices, due to their superior method performance and reliability, ability to achieve lower limits of detection (0.05-2.5 µg/kg and 0.8-30 µg/kg) and ability to capture multiple analytes in a single assay. ELISAs are more cost effective, but come with several limitations including higher limits of quantification (0.005 µg/kg) and ability to assess only one mycotoxin at a time (Marin et al., 2013; Carballo et al., 2019). We were also unable to analyze OTA in food items due to in-availability of ELISA assays at our partner analytical laboratory and cost limitations. This limited our ability to assess risk using calculated daily intake (CDIs) and estimated daily intakes (EDIs) for OTA in food. Other studies using total diets data have estimated OTA EDIs to range between 0.003-5 ng/kg bw/day (Carballo et al., 2019).

Analytical methods used for pesticides in breastmilk samples were constrained by availability of equipment at our partner laboratory in India. We were unable to use GC-MS/MS for analysis of pesticide residues in breastmilk samples, which would represent the ideal analytical method (Chen et al., 2014; Barr & Needham, 2002). Finally, as discussed previously, our LODs and LOQs for pesticide analysis are higher (µg/L), constrained by analytical sensitivity of GC-MS and ECD detectors.

7.3 Future Studies & Next Steps

There were significant limitations in our overall study design and small sample sizes. In light of findings from food sample and breastmilk analyses of mycotoxins, namely, % of positive samples and samples > maximum residue limits (MRLs) set by the Food Safety & Standards Authority of India (FSSAI), there is a need for further work in this domain. Next steps should focus on building an understanding of the extent of exposure, characterize determinants and quantify long-term impacts to the health of mothers and children, and more broadly the public, in light of the known carcinogenic effects of mycotoxins.

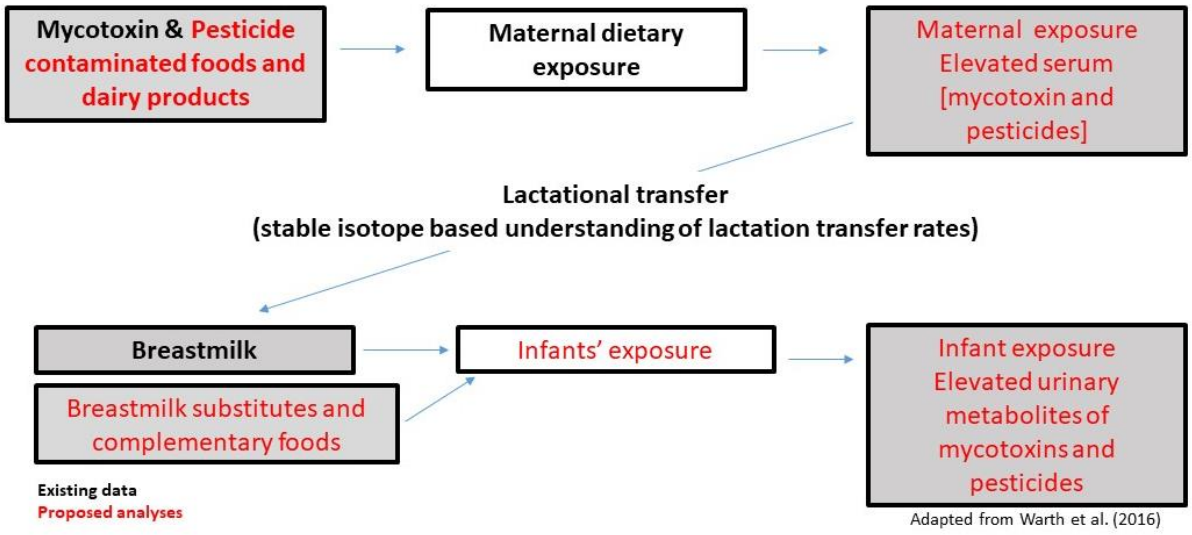
In this section, I will discuss three different potential study designs as next steps to the work presented in this dissertation. The order of this will flow as: **(1)** *immediate assessments* that can be performed with samples from the existing R21 study to answer research questions assessing the transfer of mycotoxins from mothers → infants. **(2)** Next, I will outline a design for a *longitudinal cohort study* that will allow for examination of associations between environmental exposures and child growth outcomes beginning in the pre-natal period (maternal exposure, in-utero infant exposure, lactational exposure, exposure via complementary and weaning foods and in the food system at large). **(3)** Finally, I will outline a design for potential intervention studies in line with two recent/planned *randomized control trials* in Africa that have sought to established causal linkages between mycotoxin (specifically Aflatoxin) exposure and infant growth outcomes (specifically stunting) (Hoffman et al., 2018; Phillips et al., 2020). I will also discuss the use of participatory methods for engagement of communities in such trials, in light of qualitative findings (not presented) generated as part of this dissertation work, that show poor grain storage practices in study communities, which may result in higher risk of mycotoxin contamination.

7.3.1 Leveraging existing study samples

Specific Aim: To evaluate concentrations of mycotoxins: aflatoxins, ochratoxins, deoxynivalenol and fumonisins and pesticides (organochlorine, organophosphate and pyrethroid pesticides) exposure in existing maternal serum (N = 100) and infant urine (N = 69) samples.

Additional analysis of collected and archived study samples will allow us to complete the exposure picture described in this dissertation, by assessing the transfer of aforementioned contaminants from ***maternal diet → maternal serum → breastmilk and assess lactation transfer from mother to infant during breastfeeding → infant urinary biomarkers.*** To gain a comprehensive understanding of exposure risk and transmission levels, it is essential to look at currently missing data on maternal serum and infant urinary biomarkers of exposure, as not all metabolites will be detectable in breastmilk alone. Additionally, only 28% of children in our sample were being exclusively breastfed. The remaining received water in addition to other complementary foods. Thus, the likelihood of exposure to mycotoxins via other dietary sources of exposure is high. Although we did not detect AFB_1 in infant foods in our study, levels in other food items including flour, rice and cereal grains were high (as discussed extensively in chapter 5), thus warranting further investigation of infant complementary foods.

Figure 1: Flow diagram of proposed analyses (red) to assess lactational transfer of exposures from mothers to infants



Warth et al., (2016), in a review of the state of the art in relation to the lactational transfer of mycotoxins, have highlighted a scarcity of data concerning lactational transfer rates for mycotoxins of public health interest. They note that efforts are needed to correlate maternal dietary exposure, blood plasma or serum biomarkers of exposure, and breastmilk levels. Bio-measures of mycotoxin exposure in the urine of infants could allow for combined exposure assessment for both infants and mothers, and be of particular relevance where infants are also given complementary foods in addition to breastmilk (72% of children in our sample). Given availability of maternal serum samples and infant urine samples, archived as part of the parent study, we are in a unique position to inform such correlational analyses for Biomarkers of AFs and OTs in maternal serum and AFs and OTs in infant urine. We can also assess lactational transfer rates between maternal serum and breastmilk, in addition to ingestion by the child and excretion in urine, albeit in a sample of n = 69 only.

Biomarkers of interest include, Aflatoxins: (1) **AF-alb adducts**, a measure of long-term exposure (2-3 months prior) to AFB_1 owing to half-life of serum albumin, ~ 20 days; (2) **AFB_1-lysine adducts** in plasma, the most reliable marker for chronic AF exposure; (3) **AFB-N7 guanine** in urine; (4) **AFM_1** in urine (24-48 hours) (McMillan, 2018; Vidal et al., 2018; Smith et al., 2017). These aforementioned biomarkers are validated and quantitatively associated with dietary AF intake (Smith et al., 2017). Additional AF metabolites in serum include AFM_1 and AFG_1, in urine include AFB_1 and AFG_1, and in milk include AFM1 and AFG2, however exposure levels to these have not been previously correlated to dietary intake, which is why these are referred to as bio-measures (Smith et al., 2017).

For Ochratoxins: (1) hydroxylated compounds including **7-OH-OTA, (4R)-OH-OTA**, and **(4S)-OH-OTA** in urine (Vidal et al., 2018). Biomarkers of FB1 and DON, not examined in breastmilk samples in our study can also be quantified in maternal serum samples and infant urine. Common biomarkers of DON exposure include, (1) **DON-3-glucuronide** and **DON-15-glucuronide**, suitable biomarkers for DON and DON-3-glucoside in urine (Vidal et al., 2018). Fumonisins can be detected in urine as free **FB and FB_1, FB_2, FB_3** (Cirlini et al., 2017), although FBs have low absorption and are mainly excreted via feces (Vidal et al., 2018). Given low levels of pesticides detected in breastmilk, it is also prudent to examine biomarkers of exposures in maternal serum, to validate our null-low pesticide concentration findings in breastmilk.

In the following sections, I will describe two study designs aimed at understanding different yet complementary research questions based on our preliminary pilot findings. The first proposed design will involve establishment of a longitudinal cohort using a prospective epidemiological study design. This design will help answer several questions pertaining to:

1) risk and prevalence of exposures from conception/pregnancy → 2 years of life (~ 3-year time period)
2) gestational and lactational transfer of exposures from mothers to infants
3) associations between exposures and infant growth outcomes in the first 1000-day period

The second proposed study will capitalize on existing data which suggest high levels of mycotoxin contamination within a closed-circuit food system and propose an intervention study using a randomized controlled trial design, to:

1) mitigate household exposure
2) reduce mycotoxin load in study participants
3) improve child growth outcomes

Both studies should be considered within the context of the existing geographic communities for which we have preliminary data. However, as discussed extensively above, it is also important to ensure representativeness of the populations most at risk of exposures, in future work. To this end, both the cohort study and intervention trial should be conducted across a mix of peri-urban and remote and rural communities where agriculture is extensively practiced and direct exposure risks are elevated. Additionally, it is imperative to enroll women from the lowest socioeconomic backgrounds, who do not own their own land, practice daily wage labor and come from the most marginalized castes. Such studies can be conducted in other parts of northern India, including Bihar and Uttar Pradesh, in addition to rural districts of Haryana.

Educational levels in addition to general empowerment status of women should also be considered. It is possible that women who come from the most marginalized backgrounds have to practice daily wage labor on farms to provide for their families, thus increasing risk of exposure to harmful chemical pesticides and fertilizers, in addition to dietary mycotoxins. Additionally, although difficult in terms of feasibility and follow-up, migrant laborers from impoverished parts of northern India, who come to peri-urban parts of the country in search of economic opportunities may also be enrolled. It is important to keep in mind the precarious and temporary status of such populations however, which make them transient, yet particularly vulnerable.

7.3.2 Longitudinal cohort study

Given findings from our preliminary pilot, a longitudinal cohort study which can measure pre-natal, post-natal and follow-up exposures to mycotoxins and pesticides may be warranted. Based on our current findings, a sample size of between **670 and 720 mother-infant dyads,** accounting for loss to follow-up (+20%), would be required to achieve 0.8 power (80% chance of detecting outcome, stunting (Lombard, 2014). AFM_1 is the main mycotoxin detected in our study and of primary relevance in breastmilk. More broadly, approximately 30% of children in Faridabad district, Haryana are stunted, 20% are wasted and 21% are underweight (NFHS-4).

A longitudinal study of this nature should involve collection of maternal serum and stool biomarkers of exposures to both mycotoxins and pesticides during pregnancy, in addition to biomarkers of gut integrity in stool samples and dietary and food sample data. During pregnancy, these data can be collected once per trimester to establish pre-natal environmental exposures. Post-partum, following the work of the few longitudinal studies conducted in this area (Munoz et al., 2014; LeRoy et al., 2018), samples can be collected at 2 to 3-month intervals, for the first

year of a child's life and every 3-4 months in year 2 (hence covering the 1000-day period), as described in **Table 1**.

Table 1: Biological sample collection plan for proposed longitudinal cohort study

	Trimester			**Post-partum**							
Sample	**1**	**2**	**3**	**Days 1-6**	**2-4**	**6**	**8**	**10**	**12[a]**	**18**	**24**
Maternal plasma	x	x	x	x	x	x			x	x	x
Maternal urine and stool	x	x	x			x			x		x
Breast milk and D_2O for BM volume				x - colostrum	x	x			x		x
Infants' urine (L:M test)				x	x	x			x		x
Infant stool				x	x	x	x	x	x	x	x
Maternal and child anthropometry	x	x	x	x	x	x	x	x	x	x	x
Total diets/plate ready meal or HH food samples		x	x		x	x			x		x
HH Demographics and 24-hour recall, FFQ	x	x	x		x	x			x	x	x

[a]Breastmilk sample collection contingent on mothers' feeding habits post 12-month period

Warth et al., (2016), suggest collection of several spot samples of breastmilk over a period of 24 hours for the purposes of exposure assessment, followed by pooling of samples. This method would allow the capture of diurnal variations in breastmilk composition and subsequent excretion of lipophilic exposures into milk in relation to feeds and timing. Due to logistical, time and cost limitations, we recommend that breastmilk be collected by sampling in the morning and afternoon from 1 breast, as recommended by Wu et al., (2018).

Collected serum, breastmilk and urine samples should be assayed for mycotoxins (using biomarkers noted above) and pesticides (OPs, OCs, pyrethroids and carbamates) to understand their associations with measures of intestinal inflammation and enteropathy and in turn, infant growth outcomes. Given the magnitude of a longitudinal cohort study of the type proposed here, other environmental exposures may also be examined including PCBs, PCBBs, heavy metals (lead, cadmium, mercury).

Several measures of environmental enteric dysfunction should be studied in plasma, urine and stool samples of mothers and children as described by Prendergast et al., (2015):

1) **I-FABP** to assess villous atrophy (plasma)
2) **LPS, EndoCab, sCD14 and sCD163** to study microbial translocation (plasma)
3) **AGP, CRP, Ferritin** to study systemic inflammation (plasma)
4) **IGF-1** to study growth hormone resistance (plasma)
5) **REG-1B** to assess altered gut structure and function + impaired epithelial regeneration (stool)
6) **Neopterin, myeloperoxidase and alpha-1-antitrypsin** to study mucosal inflammation (stool)

7) **Lactulose:Mannitol Ratio**, lactulose recovery to assess intestinal permeability and mannitol recovery to study reduced absorptive surface area (urine)

It is also recommended that markers of systemic inflammation including cytokines, IL-6, IL-8, IL-10, TNF-α, and acute phase proteins, AGP, CRP and ferritin be studied in both maternal serum and breastmilk (Harper et al., 2018). The advantages of a longitudinal pre-natal to 24-months of age range, with routine follow-ups, is our ability to track exposure (mycotoxins and pesticides) and outcome (infant gut integrity, infant growth – LAZ, WAZ, WFL) using a prospective epidemiological design. The proposed data collection plan would also allow us to capture seasonality for breastmilk and food samples. Disadvantages include participant burden, costs, logistics, loss to follow-up and attrition. The aforementioned study design would allow us to examine risk of exposures, gestational and lactational transfer of exposures from mothers to infants, and examine associations with infant growth outcomes in the first 1000-day period.

A cohort of this nature may be established by enrolling women attending pre-natal clinics at primary health centers (PHCs) or government/private hospitals, and/or leveraging existing cohorts established by partner implementing organizations in the region.

7.3.3 Randomized controlled trial/intervention study

Finally, a third approach would involve an intervention study to deliver mycotoxin free-wheat (flour) and rice (staple crops in the community), in addition to behavior change communication (BCC) based interventions in study communities.

Food based intervention:

These flours and rice would be milled (only applicable to flour), tested for regulatory compliance and delivered by the study team (third party vendors) to ensure quality. It is also possible to train women's groups in study communities to produce the flours, store and sell them

at a marginally low price, to build income generating potential. The study team would provide technical support to ensure regulatory compliance. Addition of other micronutrient fortificants such as iron, folic acid and zinc may also be considered.

Based on our findings described in chapter 4, AFB_1 and FB_1 levels are problematic in wheat, rice, wheat flour and groundnuts collected from study communities. Wheat and flour-based products are routinely consumed in these communities (95% of women noted consuming roti made from wheat flour; 50% of women noted consumption of rice). Formative research and a trial of improved practices (TIPS) would be necessary prior to intervention implementation to test palatability of flours and contextualize the food items to study context, examine acceptability and preferences.

Community based participatory - behavior change communication intervention (CBPR-BCC):

In addition to delivering a food-based intervention (Hoffman et al., 2018; Phillips et al., 2020), enrolled women in intervention and control groups would also be provided with training on post-harvest crop strategies informed by good agricultural practices (GAP) including proper drying, grain storage, and the use of hermetic storage technologies, using community-based participatory action research (CBPR-BCC) intervention modalities (Minkler & Wallerstein, 2003; Wenndt, 2017). Community members will be trained in active management and monitoring of contamination in their households and community, to engage them in the research process and success of the BCC component.

Kamala et al., (2018) in their cRCT used several post-harvest strategies to reduce AF and FB contamination (specifically in maize, but would require contextualization to Haryana, as maize is not a primary component of the diet in our study communities). These researchers used hand sorting (prior to storage and use), drying maize on a mat/raised platform, proper sun drying,

application of storage insecticides and de-hulling before milling. In intervention communities, mean concentrations of AFs and FBs were significantly lower ($p < 0.05$) in the intervention group compared to control, with intervention effects ranging between -4.9 (95% CI: -7.3, -2.5) to -405 (95% CI: -647, -162) µg/kg. This corresponds to an 83 and 70% reduction in AFs and FBs, respectively (Kamala et al., 2018). Similar post-harvest interventions can be contextualized to Haryana using formative research strategies and integrated into an RCT design. More recently, Wenndt et al., (2020, unpublished results), developed and delivered similar participatory action research-based interventions to reduce mycotoxin exposure in Uttar Pradesh, by engagement of small-holder farmers.

The advantage of a CBPR-BCC based approach is its ability to inform sustainable long term agricultural extension programs to improve household practices and potential community trickle down effects, where a food-based intervention would inform the efficacy of reducing exposure to mycotoxins using the specified items and associated impact on measured outcomes.

Study design and sample sizes:

A 3 x 2 factorial design approach is recommended for this cRCT, with the following groups:

1) Food based intervention alone vs. control
2) CBPR-BCC alone vs. control
3) Food based intervention + CBPR-BCC vs. control

In line with proposed RCT by Phillips et al., (2020), a sample size of 200 households or more per arm is recommended (total N ~ 800 mother-infant dyads), contingent on costs, logistics and other factors. Households with pregnant women should be enrolled (baseline) and followed up to when children reach the age of 24 months (capture the first 1000 days). Randomization should be

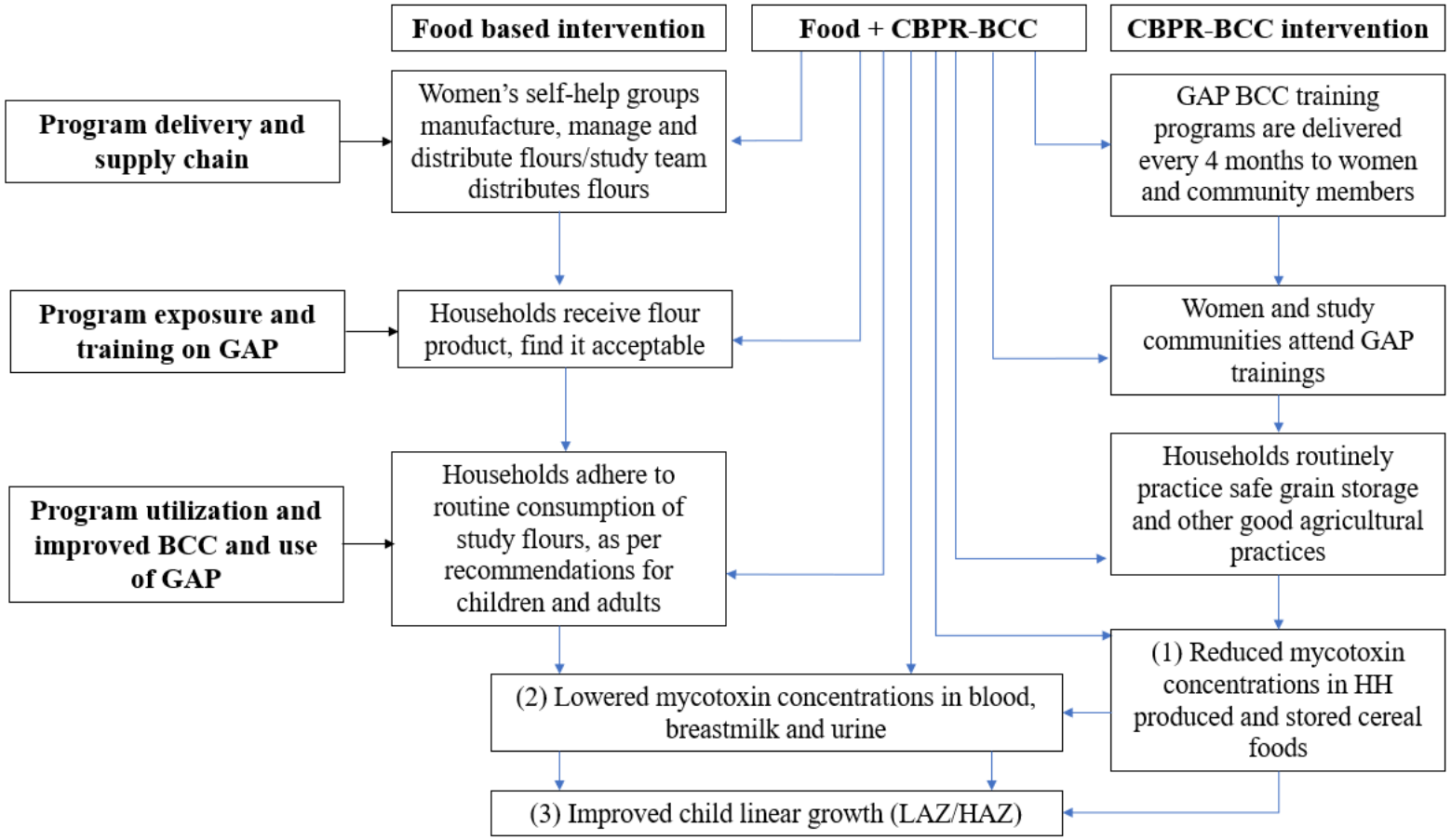
conducted at the community level, with non-intervention receiving communities serving as control group.

Intervention delivery and impact evaluation:

Intervention delivery by study team and/or trained community members (community based participatory action research methods) would occur at baseline, and every 4 months subsequently, with monitoring data collection once every 4 months as well. Midline assessment would be conducted when the child is between 6-10 months and endline at 24 months of age, with an additional biomarker collection point at 18 months post-partum. Main outcomes will include: lowered mycotoxin (and pesticide) concentrations in household food items, lower mycotoxin concentrations in maternal and infant serum, breastmilk, urine, lower L:M ratios and other markers of gut integrity in mothers and infants; improved infant LAZ. Additional data on 24-hour recalls and FFQs would also be collected, in addition to standard sociodemographic and household characteristics, maternal and infant morbidity.

Impact results (biomarker data) would be compiled at time of enrolment (trimester 1), 6-10 months postpartum and 24 months postpartum, respectively. If not leveraging the aforementioned longitudinal cohort study, collection of biomarkers of exposure can be restricted to the three time points described above, although the same women would be longitudinally followed (not cross-sectional time shots). Adherence to the intervention and implementation fidelity should also be assessed using an intention-to-treat and per-protocol analysis. The intervention can measure changes in levels of mycotoxin contamination in household storage due to the intervention, in addition to human intake of the same, with assessment of health impacts in mothers and infants. A proposed hypothetical program impact pathway is shown in **figure 2** below.

Figure 2: Proposed program impact pathway (PIP)



Chapter 8: Conclusion

We developed and validated a method using high-performance liquid chromatography tandem mass spectrometry, which was first tested in a small sample of animal milk to then quantify breastmilk mycotoxins. To the best of our knowledge, this is the first study from India to use UHPLC-MS/MS SRM to quantify mycotoxins in animal and human milk samples. Others have used less sensitive and specific methods such as HPLC-FD and ELISA for mycotoxin assessment in animal milk samples and no prior studies to date have assessed mycotoxins in breastmilk from India.

This highly selective and specific method can be used for routine testing of mycotoxins in animal and human milk samples at a low time and monetary cost (INR 3000-5000, USD 40-66), and as part of routine surveillance and bio-monitoring efforts. We found moderate to high levels of AFM_1 across a variety of animal milk samples, findings which are in line with larger studies conducted by the Food Safety and Standards Authority of India.

Breastmilk is the most important source of nutrients for infants < 6 months of age, and exclusive breastfeeding remains the gold standard for infant feeding, despite the potential for exposure to environmental contaminants such as mycotoxins and pesticides. Ours is one of only a few to have examined the issue of breastmilk mycotoxins in the South Asia region. We did detect AFM_1 in breastmilk samples quantified in our study, but only 1% was above regulatory limits. Our findings are reflective of the extant literature which has quantified this mycotoxin at varying levels, in samples from across the globe. Our reported concentrations are lower than those reported in some parts of Africa, Europe and the Middle East (Coppa et al., 2019; Fakri et al., 2019). Although we did not detect quantifiable levels of Ochratoxin A in our samples, other studies have also found this mycotoxin in breastmilk from several countries across the globe

(Coppa et al., 2019; Cherkani-Hassani et al., 2016). Results from food sample mycotoxin analyses suggest that AFB_1 is a problem in the food supply and warrants further attention. Consumption of breads was a statistically significant determinant of AFM_1 in maternal breastmilk in our study, adding further credence to the need for control of parent toxin, AFB_1 in the food system. Risk assessments for mothers and children also suggest a need for added research on the issue of mycotoxin contamination upstream, within the food system, in our study context.

Our findings regarding pesticides in breastmilk are not in line with other studies that have detected OC, OP and pyrethroid pesticides in Indian human milk samples, at levels that may impact public health. Our LODs and LOQs are higher than those seen in the extant literature. That said, our LODs are below maximum residue limits prescribed for DDTs in milk, thus allowing us to detect concentrations of public health significance. We found DDT and DDE in only a small sub-set of tested breastmilk samples. Our qualitative findings support breastmilk biomarker results. Due to gendered engagement in farming in study communities, women do not directly handle or use chemical pesticides, and are therefore at lowered risk of occupational exposure.

Finally, although we had null findings for associations between breastmilk aflatoxins, infant gut integrity and infant growth, we acknowledge several limitations in our analyses. These include age of children enrolled, sample size and lack of power. Biological plausibility for such associations exists and has been demonstrated in animal models and *in vitro* cell experiments to show the impacts of mycotoxin exposure on the intestine. Larger studies with longitudinal designs are needed to help elucidate these associations and provide support for intervention studies in our context.

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