FIRST FOODS, INTESTINAL ECOLOGY, AND EARLY LIFE HEALTH AND GROWTH OUTCOMES

by

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ABSTRACT

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Thesis directed by Dr. Robin Bernstein

Dietary exposures during early life, including our 'first foods' (i.e., mother's milk and non-breast milk foods), have lasting impacts on offspring development. In this dissertation, I explore the impact of first foods on infant health and growth outcomes in a rural population under considerable environmental and physiological stress in The Gambia.

I utilize longitudinal data and biological samples collected as a part of the Hormonal and Epigenetic Regulators of Growth (HERO-G) study, which was designed to investigate growth patterns in rural Gambian infants using epigenetic, endocrine, and metabolic analyses. I begin by describing the first foods given over the first year of life in this cohort, including characterization of breastfeeding and complementary feeding practices, and assess some nutritional and bioactive factors in maternal milk across the first year of lactation. Next, I detail infant morbidity occurrences within this cohort across the first year of life, contextualizing infant health outcomes in relationship to diet. Finally, I investigate growth outcomes using anthropometric measurements across the first year of life and test whether first foods and morbidity occurrence exert an effect on weight-for-height (WHZ), height-for-age (HAZ), and/or weight-for-age (WAZ) outcomes. As a part of these investigations, I measure infant fecal pH across the first year of life and explore the relationship of intestinal ecology to environmental stressors and dietary shifts.

I identified "real time" and "extended" effects of first foods and breastfeeding practices on infant morbidity and growth at 3, 6, 9, and 12 months of age, demonstrating the persistent contributions of first foods on offspring outcomes. Robust maternal investment in offspring through complex nutritional and immunological milk profiles provides protective effects against illness in infants in this rural Gambian environment, and fewer symptomatic morbidities are associated with features of longitudinal growth. Generating an evidence base that considers both the immediate and longer-term benefits of nutrition in early life is critical to a comprehensive assessment of pathways and underlying mechanisms connecting early life environment to later life outcomes. This project provides important insights into physiological, evolutionary, ecological, and sociocultural influences on nutrition, variation in human milk profiles, and infant health and growth outcomes.

DEDICATION

I dedicate this dissertation to my grandmother, Dr. Sheila Becker, who relentlessly encouraged me to work hard in school, just like she did. I know the end result would make you proud.

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[1] HERO-G Working Team: Field team: Saikou Drammeh, Kemo Ceesay, Kebba Bajo, Lamin Sowe, Hasum Ceesay, Ebou Touray, Amadou Touray, Lamin Ceesay, Bubacarr Jobarteh, Ansumana Bajo, Fakebba Camara, Kassa Kora, NuhaCamara, Lamin Jarjou, Sirimang Malang, Mariama Saidykhan, Muhammed Ceesay, Isatou Jammeh, Andy Doel, Saikou Darboe, Darboe Bah, Alagie Ceesay, Mariama Njai, Fatou Touray, Ousman Njai, Ebrima Ceesay, Suntu Ceesay, Kaddy Sanyang, Kawsu Ceesay, Isatou Jallow, Lamin Bajinka, Essa Touray, Lamin Jarjou, Momodou Dibba; Investigators: Momodou Darboe, Branwen Henning, Helen Nabwera, Rita Wegmuller; Lab team, MRC Unit the Gambia at Keneba: Alpha Omar Jallow, Ebrima Sise, Amadou Jallow, Alasana Saidykhan, Ebrima Bah, Alhasan Colley, Saikou Sanyang; Clinic team: Patrick Nshe, Fatou Sosseh, Ousman Jarjou, Yusapha Dampha, Jane Jackson, Edrisa Sinjanka; Data team: Mohammed Ngum, Abdoulie Faal, Bakary Sonko

grow both as a researcher and on an individual level. I am grateful to have been able to have those experiences, both the challenging and successful moments alike.

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LIST OF ABBREVIATIONS

BFCI: Baby Friendly Community Initiative
BIC: Bayesian information criteria
BMR: basal metabolic rate
CI: confidence interval
DFLNHa (Difucosyllacto-N-hexaose [a])
DFLNHb (Difucosyllacto-N-hexaose [b])
DFLNO I (Difucosyllacto-N-octaose I)
DFLNnO II (Difucosyllacto-N-neooactaose II)
DFpLNH II (Difucosyl-para-lacto-N-hexaose)
DFS-LNH (Difucosylmonosialyllacto-N-hexaose)
DFS-LNnH (Difucosylmonosialyllacto-N-neohexaose)
EBF: exclusive breastfeeding
E. coli: Escherichia coli
EE: environmental enteropathy
ENID: Early Nutrition and Immune Development
Fuc: fucose
F-LSTc (Monofucosylmonosialyllacto-N-neotetraose)
FS-LNnH I (Fucosylsialyllacto-N-neohexaose I)
FS-LNO (Fucosylsialyllacto-N-octaose)
FUT2: fucosyltransferase
Gal: galactose
Glc: glucose
GlcNAc: N-ethylglucosamine
GCC: graphitized carbon cartridges
HAZ: height-for-age Z score
HERO-G: Hormonal and Epigenetic Regulators of Growth
HMGP: human milk glycoprotein
HMO: human milk oligosaccharide
H. pylori: Helicobatcter pylori
IFLNH III (Isomer 3 fucosyl-para-lacto-N-hexaose)
IFLNH I (Isomer 1 fucosyl-para-lacto-N-hexaose)
IgA: immunoglobulin A
LDFT (Lactodifucotetraose)
LF: lactoferrin
LMIC: Low- and middle-income countries
LNDFH I (Lacto-N-difucohexaose I)
LNDFH II (Lacto-N-difucohexaose II)
LNFP II (Lacto-N-fucopentaose II)
LNFP I + III (Lacto-N-fucopentaose I + III)
LNFP V (Lacto-N-fucopentaose V)
LNH (Lacto-N-hexaose)
LNnH (Lacto-N-neohexaose)
LNT (Lacto-*N*-tetraose)
LSTc (Sialyllacto-N-tetraose [c])
LSTc (Sialyllacto-N-tetraose [b])
MCIS: Multiple Cluster Indicator Survey
MFLNH I + III (Monofucosyllacto-N-hexaose I + III)
MFpLNH IV (Fucosyl-para-lacto-N-hexaose)
MRC: Medical Research Council
MUAC: mid-upper arm circumference
NaNA: National Nutrition Agency
NBMF: non-breast milk food
NHP: non-human primate
PCA: Principal Component Analysis

p-LNH (para-lacto-N-hexaose)
PLS: Partial Least Squares
PRESS: Predictive Residual Sum of Squares
SA: sialic acid
SD: standard deviation
S-LNnH II (S-Lacto-N-neohexaose II)
TFLNH (Trifucosyllacto-N-hexaose)
TOF: time-of-flight
UTI: urinary tract infection
VIP: Variation in Projection
WAZ: weight-for-age Z score
WHO: World Health Organization
WHZ: weight-for-height Z score
2'FL (2'-Fucosyllactose)
3'FL (3-Fucosyllactose)
3'SL (3'-Sialyllactose)
6'SL (6'-Sialyllactose)
2010a; 2010b; 2010c
3120a; 3120b; 3200a; 3200b: No literature name
4100a; 4100b; 4110a; 4110b; 4210a; 4210b: No literature name
5300a; 5300b; 5301a; 5310a; 5310b; 5310c: No literature name

CHAPTER 1. ANTHROPOLOGICAL CONTEXT AND PROJECT BACKGROUND

INTRODUCTION

First foods – including maternal milk and non-breast milk foods – consumed during early life may have particularly important impacts on offspring health and growth. Existing studies show variation in breastfeeding practices around the world, along with a range of similarities and differences across maternal milk compositional profiles. Growing evidence also demonstrates complex relationships between early life dietary factors, immune function, and somatic growth outcomes in both the short- and long-term.

Given the scarcity of concurrent longitudinal data on infant feeding practices, maternal milk composition, and infant health and growth outcomes, few studies have had the opportunity to comprehensively evaluate the interrelationships between all of these factors. Examining these variables in isolation, however, may reduce the resolution of their individual and combined effects. A comprehensive understanding of the influence of early life diet on health and growth outcomes is of particular importance in low-income populations that experience marked seasonality associated with annual food insecurity, heavy maternal workload, and fluctuations in infectious disease burden. In such populations, these factors influence early life growth, the outcome of which is linked to infant/childhood – and later life – morbidity and mortality. Generating an evidence base that takes into consideration both the immediate and longer-term impacts of nutrition in early life is critical to a deeper investigation of the pathways (and their underlying mechanisms) linking the early life environment to later life outcomes.

Using a life history framework to investigate detailed infant feeding and health and growth data could offer a framework for insight into the dynamic nature of lactation as well as the nuanced impacts of first foods on infant outcomes.

This thesis aims to address the following primary question:

What are the effects of early life diet (including maternal milk composition and infant complementary feeding practices) on infant morbidity and growth during the first year of life?

In this introductory Chapter, I will discuss key anthropological perspectives on breastfeeding, including biocultural and evolutionary aspects of maternal milk and interrelationships between nutrition, infection, and growth. I begin with an overview of breastfeeding in the context of mammalian and human evolution, including a description

of infant and maternal life history traits. I then synthesize the effects of breastfeeding on infant health and growth. This is followed by a section describing some of the cultural variation in infant feeding practices around the world. Finally, I conclude by introducing the design of the HERO-G study – emphasizing the methods and collection time points of interest for this project – and describe the structure of the remaining Chapters of this dissertation.

LIFE HISTORY THEORY AND TRADEOFFS

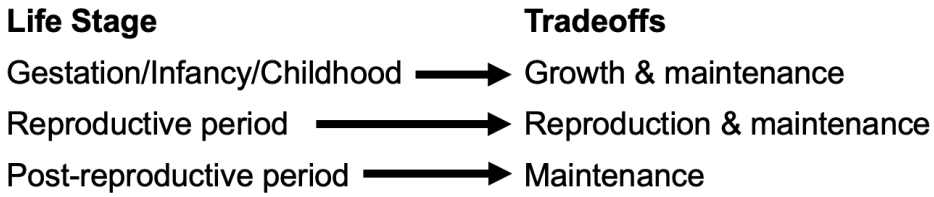
Variation fuels evolutionary change[1]. Life history theory utilizes concepts such as natural selection, fitness, adaptation, and constraints to explain variation in patterns of species' reproduction and survival. Life history traits are the collective phenotypes that influence the survival and reproduction of an organism[2]. These traits, such as the number and size of offspring, age at first reproduction, and lifespan, are situated in the context of an individual's reproductive success and the resources required to maintain the energetic costs of these traits. The Principle of Allocation helps explain how some variation in life history characteristics emerges under different environmental contexts[3,4]. Under the 'allocation theory,' energy is treated as a limited resource that must be preferentially allocated to systems – sometimes at the expense of others – that are expected to support maximal fitness[2,5]. Such allocations require coordination between multiple physiological and behavioral systems, most of which can be sensitive to moderation by environmental factors.

The concept of tradeoffs informs how we understand and assess patterns of life history over an organism's life; they direct the development of hypotheses and interpretations of results from investigations of variation in life history traits. Simply put, a tradeoff occurs when an investment in one process reduces investment in another, assuming resources available to the individual are limited. Classic life history tradeoffs include those between reproduction, maintenance, and growth, with common life history traits including size at birth, adult body and brain size, weaning age, reproductive age, interbirth interval, number of offspring, gestation length, and lifespan. The combinations of these individual traits are referred to as life history strategies.

Broadly speaking, tradeoffs can be generalized by life stage (**Figure 1.1**). During gestation, infancy, and childhood, 'maintenance' – which includes immune function, cellular repair, and organ function – is thought to compete with somatic growth for available energy resources. Because of the enormous energetic costs of reproduction, the start of the reproductive period typically coincides with the cessation of the similarly costly period of growth[1,2,6,7]. In the reproductive stage, tradeoffs are seen between maintenance and reproduction, particularly under circumstances

of limited energy intake or increased energy demands[8–12]. Where a post-reproductive life stage occurs, energy is focused on maintenance as opposed to processes related to reproduction.

Figure 1.1. Classic tradeoffs associated with stages of human life history



Optimal energy allocation strategies can differ, in part, based on variation in ecological factors, such as food availability, mortality hazards, and local disease patterns[13,14]. For example, healthy individuals allocate energy differently from those combatting an infectious disease. Energetic allocations towards immune function when living in stable, predictable conditions may vary compared to settings with unpredictable future circumstances (e.g., food insecurity, seasonal infectious disease load). Energy allocation is also age-dependent, with newborns allocating energetic resources differently from adults. In particular, environments with high risk of mortality place precedence on energy allocation towards survival and reproduction over growth and maintenance[2,5,12,15–17].

Life history strategies can be viewed as a spectrum from 'fast' to 'slow'. Humans are categorized as having mostly slow life history characteristics: gestation is long and generally results in singleton births, we have an extended childhood life stage, later age at first reproduction, larger adult body size, and longer lifespan. In the order Primates, which includes humans, the timing of some life stages are prolonged with increases in body size[13,18–23]. For example, compared to most other mammals, primate offspring generally grow slowly and reach reproductive age relatively later in life[16,19,24–26]. Humans produce offspring that are secondarily altricial (or semi-precocial), where infants are born in an underdeveloped state compared to other non-human primates and require additional care and feeding from another individual[13,21,27]. Trevathan and Rosenberg (2016) further describe human neonate altriciality as including large body size relative to other apes, small brain size relative to the human adult, and a prolonged period of motor immaturity compared to other apes[11].

Lactation, unique to mammals, is the primary vector of energy acquisition and determinant of energy availability for newborns and infants and is a major reproductive investment on the part of the mother. For mammalian species, early infancy represents a unique period within the life cycle in which a single food source – maternal milk –

can meet the dietary needs of developing offspring. In this way, maternal milk is the major source of energy allocated during infant development that drives tradeoffs until energy is introduced from non-breastmilk nutritional sources. After cessation of breastfeeding, human diets are quite varied[28]. Maternal life history strategies such as lactation are a rich source of phenotypic plasticity; the finer details of milk constituents and complementary feeding practices are directly relevant for adjustments made by offspring that carry long-term implications for offspring health, developmental trajectories, and survival[29–31]. After delivery, maternal energy can be allocated to current or future offspring, a 'decision' which can be mediated by lactation and timing of introduction of non-breast milk foods. Tradeoffs and energetic investments by both mother and offspring, and the interaction between the two, are important to consider when investigating questions from a life history framework[32,33]. I discuss these topics in the following sections.

Infant life history

Infancy has been broadly defined as the life stage between birth and cessation of breastfeeding[34]. In hunter-gatherer (forager), horticultural, and agropastoral populations, the complete cessation of breastfeeding generally occurs around 24-36 months of age[35–38]. The infancy period can be characterized by breastfeeding, the presence of deciduous ("milk") teeth, introduction of non-breast milk foods, rapid growth and rapidly decelerating growth velocity[39,40]. Much research has been conducted characterizing early life tradeoffs between growth and maintenance through an evolutionary framework.

Growth

Infant growth is characterized by the fastest growth rate of any postnatal stage, with the earliest days of infancy representing a continuation of fetal growth velocity. Growth rate rapidly decelerates throughout infancy until beginning to plateau in childhood. This is possibly related to breastfeeding, which is present cross-culturally and may outweigh or buffer against environmental or other exogenous factors (e.g., socioeconomic position)[41].

Infant growth is a dynamic and energetically costly process. It is directed by genetic, endocrine, and nutritional factors, and is also sensitive to environmental influence, such as from factors correlated with seasonality or socioeconomic position (e.g., fluctuating disease burden, lack of access to health care resources, or lack of access to clean sources of drinking water, etc.)[42]. Human infants diverge from most other mammals, including other primates,

in that significant amounts of body fat are deposited in utero[43–46]. A considerable amount of energy is devoted to fat deposition, as well as to brain growth and development, during early life. Newborns devote around 70% of growth expenditure solely to fat deposition in the first few months of life, followed by a decline during the childhood period[20,43,47].

Infants must invest in growing tissues and organs at different rates, while also maintaining those same tissues over time. In particular, the metabolic needs of the relatively large human brain may require certain adaptations to sustain its energy demands[47–50]. Newborns use around 87% of their resting metabolic rate for brain growth and development[47]. The brain grows more rapidly during this stage than other organs or tissues, such as dentition, reproductive organs, or total body mass.

Layered on to these expenditures is the cost of mounting immune responses, which are essential for infant survival, but may necessitate complex allocation decisions to promote survival to reproductive age. As an energy store, fat can buffer an infant against tradeoffs with growth, such as when energy is needed for immune activation[43,51–55]. Because infectious disease is a leading cause of infant mortality and because of the link between undernutrition and infection, adiposity/energy storage as a protective measure to compensate for an infant's immature immune system would be advantageous[56,57]. There is evidence in humans (across various ages) that fat may be specifically stored in locations where it may be important for supporting energetic costs of maintenance[51]. For example, higher pathogen load was associated with reduced central (but not peripheral) skinfolds in more than 100 different human populations, suggesting that central adiposity is more closely linked with immune system function[58].

Immune function

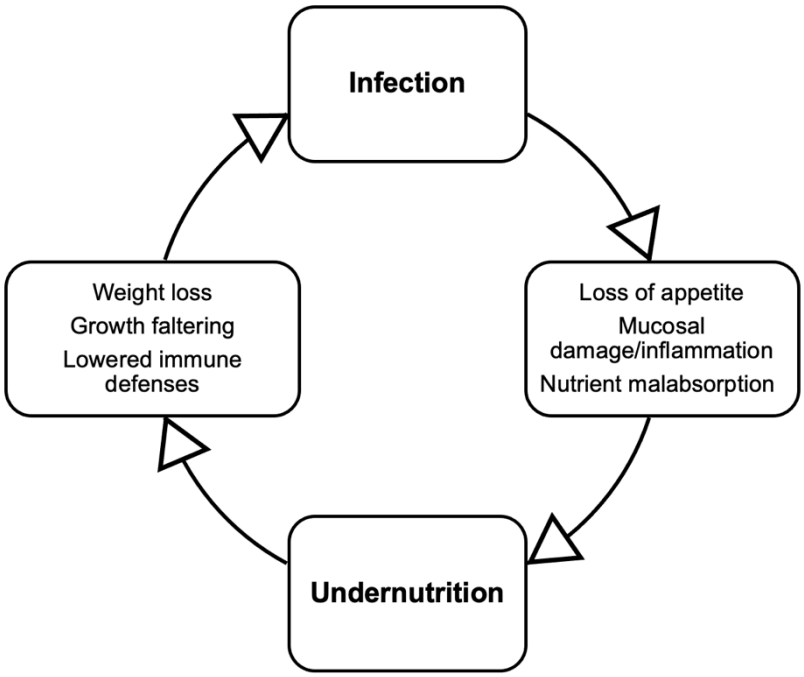
The immune system is comprised of interrelated molecular and chemical systems whose primary function is to protect the host from pathogens[59]. Its goals are to distinguish "self" from "non-self" and to adequately adapt to evolving infectious threats[59]. Humans have two systems of immunological defense: the innate and acquired immune system. There is evidence that all cellular components of innate and acquired immunity are present in the developing fetus, and the distinctions in the functionality and specializations of these two programs may relate to differences in immunological demands in utero versus in the postnatal environment[60]. The innate immune system provides broad, nonspecific immunologic defenses in response to conserved features of pathogens. These particular immune defenses are functional at birth and do not require prior exposure to a pathogen. The acquired immune system is the second

layer of immune defense and is not fully in place at birth; instead, this line of defense is characterized by specific immunological responses to pathogens that an individual has been exposed to previously. Although the acquired immune system requires more time to respond to a foreign pathogen (a specific set of defenses need to be mounted based on specific features of the pathogen), it can archive immunological 'information' from prior exposures, a capability often referred to as immunological memory. This ability can more readily protect the individual from future infections.

Maintenance is thought to compete with somatic growth for available energy resources during early life. Energy has yet to be allocated towards reproduction, thus the individual must maintain its body and continue to grow while allocating sufficient energy to defense mechanisms, such as inflammation, to ensure survival to reproductive age. For example, acute weight loss and/or growth faltering can result as a consequence of persistent and systemic inflammation during infancy and childhood, which are common in low-income countries[51,61]. The relationship between growth and immune function is detailed below.

Mounting immune defenses in response to illness or infection has significant bioenergetic and metabolic costs[62]. Immune system activation requires coordinated action by a wide range of cell types, which require an array of nutrients in order to function[15]. Due to their involvement in supporting growth and activity of immune cells, producing antibodies, and serving as their own independent antioxidants to protect existing healthy cells, a deficiency of a single nutrient (such as proteins, zinc, iron, folic acid, and/or vitamins A, C, D, and E) can alter immune function[63–65]. Additionally, illness can reduce infant appetite and disrupt caregiver feeding practices, thus reducing infant dietary intake and, by extension, the energy available for allocation[66–68]. As such, repeated episodes of morbidity can lead to poor nutritional status, and undernutrition can increase susceptibility to subsequent infections (**Figure 1.2**). Even in the absence of overt illness, chronic immune activation is energetically expensive[32,51,69–71]. The extent of damage resulting from the complex mechanisms of the undernutrition-infection cycle depend on factors such as the severity of the deficiency, functions and interactions of the deficient nutrient(s), prior history of infection, and age[61,65,72,73].

Figure 1.2. Cycle of undernutrition and infection



The immune system also includes host protection through physical barriers[59]. The gut plays an important role in this component of the human immune system. As it is constantly interfacing with external factors through diet, the landscape of the gut must adapt to meet the different functional demands imposed on it while also serving as a central site of immunological communication throughout the body. The ecology of the gastrointestinal (GI) tract is influenced in many ways by the relationships between food and gut microbiota (the collection of microorganisms – including bacteria, archaea, eukaryotes, and viruses – that inhabit the digestive tract) composition. For an infant, the gut can be viewed as a site where maternal milk can communicate with infant cellular systems and contribute protective effects. It is also the site of particular vulnerability to infection while the immune system is still developing.

A healthy GI tract functions, in part, as a physiologic barrier to prevent bacteria and endotoxins from reaching circulation[74–76]. The mucous layer, epithelial cells and their tight junctions, and gut associated tissues comprise the barrier. Impairment of this barrier can lead to severe health consequences. Immune activation interacts with nutrition to influence growth, in part, through chronic inflammation in the GI tract[77,78]. This damage, a condition often referred to as environmental enteropathy (EE), can increase intestinal permeability, allowing large macromolecules to be released into the bloodstream, and can also reduce the concentrations of important digestive enzymes. Enteropathy

broadly refers to any pathology of the intestine, whereas EE is a syndrome of inflammation related to exogenous/environmental stressors such as is seen in low-resource settings. EE is hypothesized to be caused by chronic exposure to fecally contaminated food and/or water and generally poor sanitary conditions, which are common in settings of poverty. EE was originally referred to as "tropical enteropathy" due to high prevalence across the tropics (and thus assumed to be caused by climate); however, geographical comparisons of intestinal permeability across 14 different countries showed that while EE was present across the tropics, it was absent in some tropical areas with high socioeconomic status[79]. This supports the idea that the abnormalities associated with EE are dependent upon socioeconomics and resource availability and not the tropical climate.

Bacterial overgrowth in the small intestine has been linked to EE, which is reported in high rates in children from low-income populations[78,80]. Chronic exposure to fecal pathogens is hypothesized to result in chronic inflammation capable of causing structural changes in the small intestine. Overall, EE causes a breach of non-specific host immune defenses and triggers both innate and acquired immune responses. Consistent activation of immune defenses, particularly in the intestines, can subsequently lead to functional changes such as gut barrier disruption, carbohydrate malabsorption, and continued chronic inflammation (as evidenced by biopsies and abnormal sugar absorption tests)[78]. The immunologic functionality specific to the small bowel may also be significantly impacted. Such consequences can also contribute to growth faltering and impaired development in childhood. The development and adaptation of the mucosal immune system to local environmental stressors and disease ecology is an important process during early life and has implications for longer term outcomes[81].

Cycles of undernutrition and increased susceptibility to infection are common in food insecure regions experiencing heavy disease burden. During early life, this combination can result in growth stunting[80,82,83]. The WHO categorizes children who are stunted as those whose height is < -2 standard deviations (SD) below the average for their age group (indicated by WHO defined height (or length)-for-age Z-score [HAZ] < -2SD)[84]. Percentiles and Z-scores are widely used to display and interpret growth measurements and assess nutritional status in infancy, childhood, and adolescence. These values are derived by comparing individual growth measurements against growth data from a reference population. The weight-for-height Z-score (WHZ) allows an assessment of weight in relationship to body height. Those with low WHZ (< -2SD) are considered 'wasted,' a condition that can be indicative of acute undernutrition. This is particularly true in children, whose body weights can change rapidly[85]. The process of wasting is often a consequence of insufficient food intake or high incidence of infectious diseases (particularly diarrheal

diseases). Infants with adequate nutritional status (as indicated by WHO defined weight-for-height Z-score [WHZ] > -2SD) have less severe infection episodes and reduced risk of mortality compared to those of poor nutritional status[86,87]. Weight-for-age Z-score (WAZ) indicates body weight for age and is an important indicator of nutritional status, such as underweight or overweight. Those with a WAZ < -2SD are considered underweight. By improving nutrition, the immune system could be strengthened through increased availability of key nutrients, improved appetite, and even favor growth of certain gut microbiota that support immune defense through anti-inflammatory mechanisms[61,88–92].

Longitudinal studies of growth outcomes in populations with high morbidity conditions provide evidence of both the commonalities and the subtle variation in infant growth outcomes. Evidence suggests that weight gain is prioritized over skeletal growth and height attainment in some populations[93–95] and fat accumulations are stored in locations where it may be important for fueling immune system defenses[51,93]. As one example of these tradeoffs in childhood, Tsimane forager-horticulturalists from the Bolivian Amazon, show high daily energy expenditures and resting metabolic rates[96] and Tsimane children have high levels of C-reactive protein and other markers of inflammation, which is consistent with immune responses to high pathogen burden[97–101]. Throughout early and mid-childhood, Tsimane growth is characterized by slow height velocity[102,103]. There is evidence of delayed growth spurts in some Tsimane and adult statures in this population are also short. This pattern of growth may be useful under circumstances of heavy disease burden and repeated episodes of morbidity, whereby energy could be diverted towards immune function and repair as opposed to somatic growth[104]. Others have found evidence that inflammation suppresses the growth hormone-IGF axis, with one study showing that children with higher levels of CRP between 6 weeks to 12 months of age had lower levels of IGF-1 during the same time period; both factors were associated with increased odds of stunting in the same cohort[105]. Additionally, in a cohort of children from Uganda who were experiencing severe acute malnutrition, MUAC was significantly associated with thymus size (which relates to immune function), suggesting that impacts on growth or recovery of the thymus size correlates with the recovery of muscle mass[106]. Atrophy of the thymus in undernourished children is consistently reported in the literature[107–110], further evidence of associations between growth and immune function.

Maternal life history

Maternal reproductive success is contingent on current offspring growth, health, and survival during early life. As such, maternal investment in current versus future offspring can be a major determinant of offspring fitness, and is a key component of life history strategies.

Pregnancy and fetal growth

Female reproductive output is restricted by capacity of direct metabolic investments[5,111]. During pregnancy, tradeoffs can occur between maternal maintenance and fetal growth. Pregnancy increases the risk of maternal undernutrition due to increased nutrient requirements and it can lead to micro- and macronutrient deficiencies if dietary intake is not increased accordingly. Malnutrition, including both over and undernutrition, during pregnancy can be detrimental for maternal, fetal, and neonatal health[112]. Neonatal mass and gestation length scale allometrically with maternal weight (though there is some variation between species with altricial versus precocial offspring)[5,113]. Additionally, there is an isometric association between both neonatal brain mass and maternal basal metabolic rate (BMR) with maternal weight[111]. This evidence suggests a tight link between maternal BMR and investment in offspring during gestation[5].

Fat accumulation before and during pregnancy is the primary source of energy for lactation. Around 40-50% of energy in maternal milk comes from triglycerides (derived from maternal diet), or from stores of body fat[5,114]. Maternal adiposity is related to the efficiency of lactation, whereby it is "cheaper" for mothers with high body fat to divert resources into milk composition relative to thinner mothers because a lower fraction of total energy store is required[5,115]. Maternal nutritional status can be estimated by using anthropometric indicators such as body mass index (BMI), triceps skinfold thickness (TSF), and mid-upper arm circumference (MUAC). BMI is an individual's weight in kilograms divided by the square of height in meters. A 'normal' adult female (non-pregnant) BMI range, which is based on a WHO reference standard, is between 18.5-24.9, and the CDC recommendation for weight gain for those with pre-pregnancy BMI within the normal range is around 25-35 pounds[116]. However, BMI is an imperfect measure of body fat content as it does not account for factors such as muscle mass, bone density, or edema[117]. TSF is determined by measuring the triceps skinfold on the posterior midline of the right upper arm (between the acromion and olecranon process) using calipers and is a useful marker of upper arm muscle composition (which is an indicator of nutritional

status) when used in combination with MUAC. MUAC is the circumference of the mid-upper arm, measured at the mid-point between the acromion and olecranon processes[118]. This measure is used to screen for underweight. Evidence suggests that MUAC is significantly correlated with, and thus a reliable surrogate for, BMI in assessing nutritional status in pregnant women[119–122]; however, there is no official international standard cutoff range to flag undernutrition using MUAC. The Sphere Handbook (Minimum Standards in Humanitarian Response) reports that the cutoff for undernutrition ranges from < 21 cm to < 23 cm in different countries[123,124]. Because the arm contains both subcutaneous fat and muscle, changes in MUAC can be interpreted to reflect a change in muscle mass, a change in subcutaneous fat, or both. In resource-limited settings, where individuals tend to have the lowest amounts of subcutaneous fat, changes in MUAC are more likely to reflect changes in muscle mass. This can indicate malnutrition, particularly underweight. There are other methods to measure maternal malnutrition during gestation, such as assessing maternal iron-deficiency anemia, which impacts around 42% of pregnant women worldwide[125]. Maternal anemia can lead to reduced levels of hemoglobin, which can limit oxygen availability and nutrient delivery to the fetus, and is associated with reduced birth weight and high risk of maternal mortality[125].

Maternal undernutrition during pregnancy can increase a mother's risk of morbidity and mortality. It can also increase the risk of intrauterine growth restriction, resulting in small for gestational age infants and also preterm births[126]. Low maternal MUAC measurements during pregnancy are linked with low birth weight, which is significantly associated with high rates of morbidity, growth stunting, mortality, and poor cognitive development during early life[127–132]. This suggests that maternal nutritional status may have long-term impacts on offspring outcomes. Recent studies report that around 97% of low birth weight babies are born in low- and middle-income countries, settings where estimates of gestational age can be difficult to ascertain[133]. Low birth weight include preterm neonates (born < 37 weeks of gestation), small for gestation age at term, and the combination of the two (preterm, small for gestation age neonates)[125]. Preterm births account for around one third of low birth weight infants and small for gestational age/ intrauterine growth retardation accounts for around two thirds of low birth weight infants.

Lactation and investment in current versus future offspring

Mothers also navigate tradeoffs between investment in current offspring and future reproduction. Investing in current offspring health and growth support infant survival, but simultaneously reduces the magnitude of investment in future reproduction (along with maternal maintenance)[134,135]. Milk synthesis is among the most critical and costly

features of maternal energetic investment in mammalian offspring[33,136,137], and infant feeding decisions and practices are therefore at the crux of the key tradeoff of maternal investment in offspring through breastfeeding.

In well-nourished human populations, the caloric cost of lactation is estimated around 630-670 kcal/day[9,138]. Poor nutritional status or periods of depleted maternal energy resources may induce tradeoffs between provisioning with mother's own milk or other foods, or between resource allocation to milk and investment in mother's own self maintenance. To meet the energetic costs of reproduction – including lactation – mothers can employ different adaptive strategies, such as increasing food intake, mobilizing tissue stores, increasing metabolic efficiency, or reducing overall energy expenditure[137,139]. Longer durations of exclusive breastfeeding can enhance offspring survival potential and reduce certain maternal health risks. However, shortened breastfeeding periods may reduce interbirth intervals, increase fertility rate, and reduce depletion of maternal energy reserves[41–44].

Complementary feeding and investment in current versus future offspring

Because milk production comes at an energetic cost to the mother to the benefit of the infant, the breastfeeding system itself – and in particular the nutritional and immunological aspects – may represent an example of 'parent-offspring conflict'. The parent-offspring conflict hypothesis is driven by tradeoffs between reproductive, growth, and maintenance systems. It predicts that maternal investment in current offspring will decrease if it is in the interest of future reproduction. The strategy which maximizes maternal reproductive success is likely different from the strategy that would maximize current offspring fitness[12,140,141]. Because of this, Trivers (1974) proposed that children will try to elicit as much parental investment as possible in order to maximize their own reproductive success and survival. Weaning has been used as the classic example of parent-child conflict, with the idea that increased offspring distress serves as a proxy of resistance to weaning[142]. Mothers may reduce the frequency of breastfeeding (or cease breastfeeding entirely) in order to improve their own reproductive fitness by conceiving other offspring, or to accommodate other physiological systems related to self-maintenance. Children may resist the reduction in maternal investment by engaging in "psychological warfare" (e.g., crying, temper tantrums, exaggerated dependency) to obtain additional parental investment. Relative to the reproductive interest of the mother and age-specific mortality risk of offspring, resource investment is time sensitive and depends on maternal reproductive efforts[143]. Some research suggests that a decrease in maternal investment often occurs before it is in the best reproductive interests for the infant,

and the optimal time for weaning is likely to be later for infants than for mothers. Thus, these demands lead to conflict[140,144–148].

Resistance to cessation of exclusive breastfeeding may also be influenced by many other context dependent factors. For example, a 3 month old infant who solely relies on maternal milk as their source of nutrition would be expected to resist weaning to a much greater degree than an older child whose energetic needs cannot be met through breastfeeding alone[144]. Altmann (1980) suggests that cooperation and compromise are components of resolving instances of parent-offspring conflict[149]. Others have also criticized that the parent-offspring conflict hypothesis solely focuses on the "battleground" as opposed to conflict resolution[150]. Bateson (1994 & 1995) proposed that conflict may not be a universal occurrence during the weaning process.

The "weanling's dilemma" refers to the period of transitional feeding during early life, when maternal milk alone cannot meet an infant's nutritional needs for growth, but nutritional supplementation by complementary foods increases the risk of infection and/or undernutrition[13–15]. For example, infant survival may be reduced when the energy acquired through independent foraging is insufficient to meet requirements for growth and somatic maintenance[151–153]. If introduced foods are of poor nutritional quality, it may lead to undernutrition or growth faltering. For humans, weaning too early (<6 months of age according to WHO guidelines) can also lead to delayed neurological development and increased infant morbidities such as risk of obesity, type II diabetes, and gastrointestinal, respiratory, and dermatological infections[154–159]. In hunter-gatherer, horticultural, and many low-income populations, poor infant nutrition and infection are the main causes of infant mortality[160,161].

Life history and infant feeding patterns

Infant feeding patterns are influenced by other life history features. For example, larger mammals generally tend to have longer lactation durations[162]. Primates have a particularly long lactation period. Based on body size, the expected lactation period of a primate weighing around 2.5 kg is 69 days, but observations report a duration of 166 days[137,163]. Larger bodied primates like the great apes tend to cease exclusive breastfeeding later than small bodied primates such as strepsirrhines. For example, gorillas wean between 3-6 years of age, chimpanzees and orangutans wean offspring around 5-7.7 years of age on average[35–40], but juvenile lemurs are fully weaned around 5 months of age[164]. The "ape's dilemma" described by Lovejoy (1981) suggests that the long breastfeeding duration and late weaning age practiced in non-human ape species improves offspring survival potential, but restricts future population

growth. Using complementary foods allows mothers to conserve energy, and also allows for non-maternal care strategies whereby infants can be fed by any individual, encouraging alloparenting, energy conservation, and social bonding and development[165–170].

This pattern of longer exclusive breastfeeding practices is not observed in all primates. For example, callitrichid primates (marmosets and tamarins) have relatively short lactation periods of around 75 days, though weaning begins around day 30[171]. Additionally, it is common for infants of these primates to be carried and fed by the father and other group members for around half of the lactation period[172]. Humans follow a similar derivative feeding pattern, with shorter relative exclusive breastfeeding periods along with significant investment from other individuals.

Several proposed primate traits were used to predict expected duration of breastfeeding for humans[151]. Predictive traits included tripling or quadrupling birth weight, reaching one-third of adult body weight, gestation length, and age at first molar eruption. Following these calculations, the average human weaning age was predicted to fall between 2.5-7 years of age, with most values hovering around 6 years[151]. This range perhaps illustrates the variation that may be expected of past populations. A recent metaanalysis of global human infant feeding practices showed that the combined prevalence of breastfeeding to two years of age was 33%, with considerable variation across different countries[173]. For example, breastfeeding prevalence was around 1% in Iran but over 90% in Bangladesh. Thus, human breastfeeding cannot be generalized. Instead, we must investigate population-level patterns in greater depth to identify nuanced tradeoffs.

Breastfeeding and human evolution/life history theory

Millions of years of evolution have shaped the constituents of mammalian milk, including its ability to provide a complete form of nourishment, hydration, and immune protection for offspring during early life[174,175]. Here, I will provide a broad overview of macronutrient and bioactive composition of human milk as it pertains to this dissertation.

Milk macronutrients

Milk macronutrients include fat, protein, and carbohydrates. These nutrients vary within and between mothers and across lactation, but evidence suggests macronutrient components are generally conserved across populations despite variations in maternal nutritional status and environmental pressures[176,177]. The ‘maternal

buffering hypothesis' posits that human lactation physiology can buffer milk from effects of undernutrition through the mobilization of maternal body reserves[178].

General trends in compositional shifts of milk macronutrients in humans include a gradual decline in protein and lipids over the first 6 months of lactation and an increase in lactose from colostrum and transitional milk to mature milk[138,153]. Requirements for macro- and micronutrients are higher during infancy relative to any other life stage due to the rapid cell division that characterizes growth, which requires energy, protein, and nutrients that are involved in DNA synthesis.

Relative to body size, energy needs are greater in infants compared to adults. An infant's resting metabolic rate is around two times greater than in adults. Between 0-6 months of age, infants require around 450-650kcal/day and around 600-850kcal/day between 6-12 months of age[179]. The WHO reports that mother's milk can provide half or more of a child's energy requirements between 6-12 months of age, and around one third of requirements between 12-24 months of life[180]. The principal carbohydrate in human milk is lactose, which is the least variable of the macronutrients. Around 40% of the calories in human milk are provided by lactose. Thus, caloric content of maternal milk and its abundance of certain constituents is an important contributor to infant growth.

Human milk oligosaccharides and glycoproteins

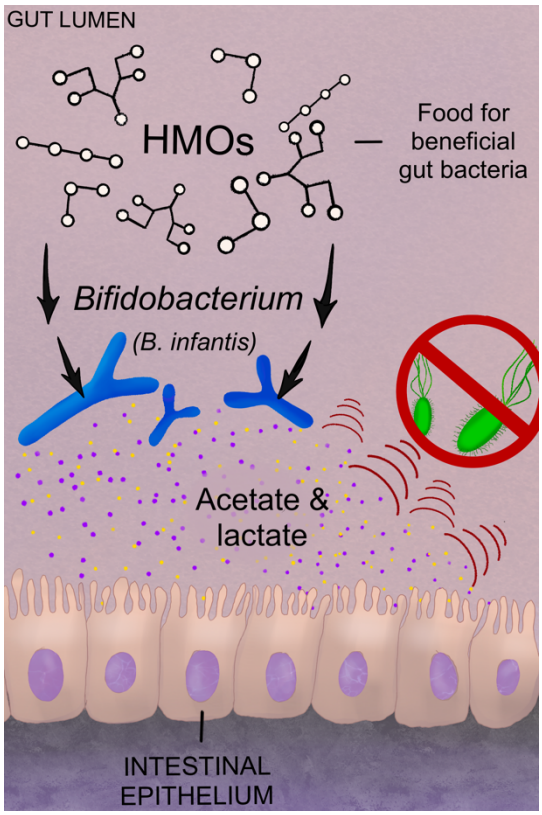
The second most abundant carbohydrates are human milk oligosaccharides (HMOs), which are among the non-nutritive bioactive factors found in maternal milk. Milk bioactives are components that impact biological processes or substrates and subsequently influence body function, condition, and health[181]. HMOs are a structurally and biologically diverse group of sugars which are indigestible to the infant. HMOs can be grouped based on their varieties of complex structures, the synthesis of which largely depends on maternal genetics. Mothers with the active gene for fucosyltransferase (*FUT2*), are referred to as "Secretors". Mothers without the functional *FUT2* enzyme (non-Secretors) produce milk without (or in very limited abundances) 1,2-fucosylated (2'FL) HMOs. As such, the abundance of 2'FL in maternal milk is an indicator of Secretor status.

In addition to HMOs, human milk glycoproteins (HMGPs) also represent bioactive factors in maternal milk. HMGPs contain sugar chains structurally similar to HMOs[182–184], and they are less digestible and of less nutritional value than other protein types[185–187]. Among the most abundant HMGPs are lactoferrin and immunoglobulin A (IgA). These bioactives have been shown to compensate for deficiencies in the neonatal immune system. For example, they

can introduce an immunological memory to offspring, allowing the infant immune system to recognize and destroy pathogens without prior direct exposure[188,189]. Lactoferrin and IgA both have antimicrobial and anti-inflammatory properties which provide passive immunity to the infant while their immune system develops. Both also play roles in directly and indirectly protecting neonates against infections caused by a variety of pathogens. They have demonstrated capabilities in the prevention of diarrhea, necrotizing enterocolitis, and neonatal sepsis[190]. Both HMOs and HMGPs play key roles in establishment and maintenance of commensal gut bacteria[191,192] and can inhibit pathogenic bacteria from adhering to the surface of intestinal walls[193–196]. They also serve as substrate for the immature infant gut microbiota, fostering growth of key gut microbes such as *Bifidobacterium*[191,197–202].

The beneficial gut microbe *Bifidobacterium* consumes HMOs and HMGPs and produces organic acid byproducts (acetate and lactate)[203–206]. This subsequently lowers intestinal pH, creating an inhospitable gut environment for many pathogenic bacteria, but fostering further growth of bifidobacteria (**Figure 1.3**)[175,207]. Loss or absence of bifidobacteria in the gut is marked by elevated GI pH[208,209]. Increased GI pH may indicate perturbation of microbiota, which may lead to chronic inflammation and morbidity[209]. In food insecure populations experiencing heavy disease burdens – particularly of GI-origin – maternal milk and infant gut microbes may be crucial mediators of morbidities and drivers of longer-term health and growth outcomes.

Figure 1.3. HMO and gut interaction example



Despite having little to no nutritional role to the infant, maternal energy is allocated into HMO and HMGP production throughout the course of lactation. Infants with certain gut microbiota profiles – particularly those dominated by *Bifidobacterium* – may be capable of optimizing maternal milk bioactives for improved health and growth. Beyond this, the nutrients and immune factors transferred through maternal milk affect offspring phenotype, making it a potential source of epigenetic inheritance. In fact, evidence suggests that maternal effects are adaptations that facilitate offspring phenotypic responses to environmental pressures and local disease ecology based on exposures over the mother's lifetime[188,210]. While the infant immune system develops, maternal milk bridges the gap between individual tradeoffs in herself and her offspring; this combination creates a "pooled" life history strategy (**Figure 1.4**).

Figure 1.4. Maternal & infant (A) individual and (B) "pooled" life histories

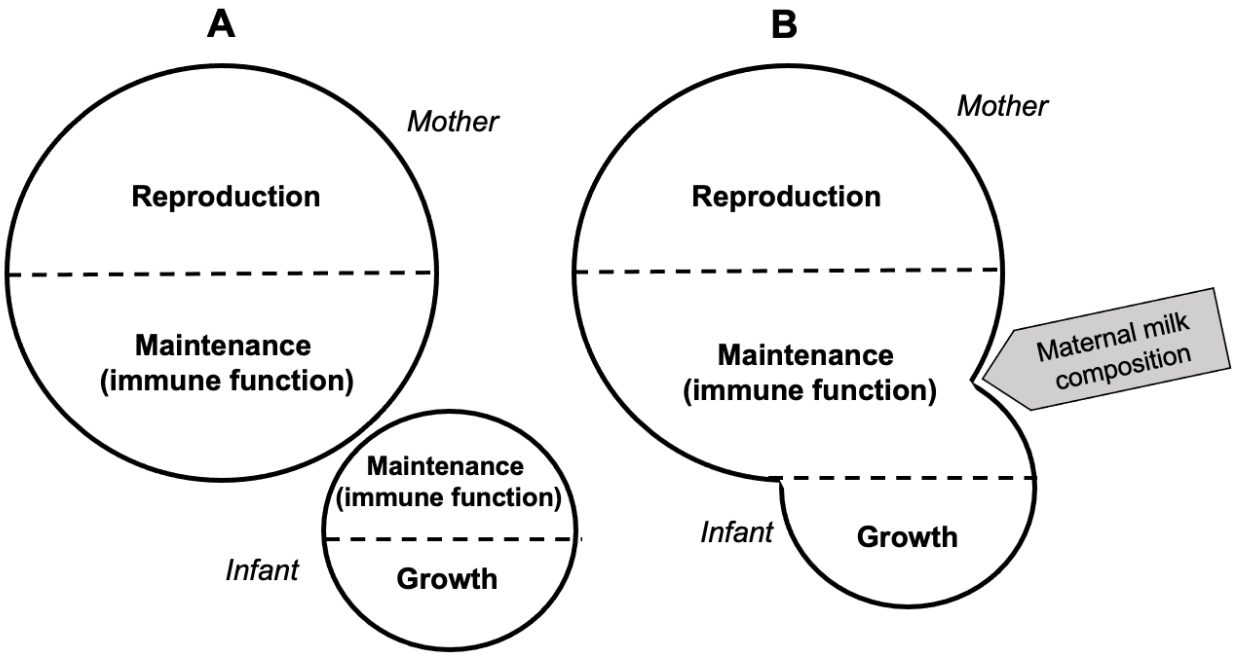


Figure adapted from Gowland (2019) *The Mother-Infant Nexus in Anthropology*[211]

Ultimately, early life experiences and environments, including nutritional environments, can have profound effects on offspring phenotype[2,212,213]. The early postnatal period represents the window of maximal expression of adaptive plasticity in postnatal life because developing infant physiology is highly sensitive to pressures from environmental and nutritional factors[214–216]. There is strong evidence that nutrition, growth patterns, and environmental factors during early life are linked to physiological and epigenetic mechanisms that result in phenotypic modifications with negative outcomes such as non-communicable disease[217–219]. For example, there is evidence that infants who experience nutritional insult during early life have impaired immune systems as adults[215]. Additionally, reduced maternal investment during the course of lactation can result in increased infant morbidity and mortality[220].

Variation in infant feeding practices globally

Cultural factors play a large role in human biological outcomes related to nutrition in general and breastfeeding in particular. Cultural attitudes regarding the roles of women and mothers, child development and care, diet, medicine and maternal milk itself shape infant nutrition in a variety of ways. Food itself offers an insight into

cultural factors influencing the weaning process. Despite wide variation in adult diets worldwide, there are certain broad patterns associated with the feeding of supplementary foods.

Beliefs about food can shape dietary behaviors[221]. Food taboos can impact the amount, frequency of consumption, and quality of nutrients in one's diet[222]. Older studies of maternal perceptions and factors influencing child feeding decisions in The Gambia have shown that initiating breastfeeding after delivery is generally delayed due to cultural practices[223], and many women reported that colostrum ('first milk') was perceived as impure and unsafe for infant consumption. For example, under certain cultural beliefs, colostrum is viewed as "bad milk" because its off-white to yellowish color resembles pus, or is seen as non-nutritious, and is discarded while infants are fed alternatives such as sugar water or non-human milk[224,225]. In 2001, less than 8% of Gambian mothers (N=324) used wet nurses to breastfeed their babies in place of their own colostrum[226]. Koranic potions, made from juice of the kola nut (a caffeine-containing nut from evergreen trees), are given to some infants instead of colostrum, and some rub kola nut or salt under the infant's tongue, which is thought to help the child's speech later in life[224]. Liquids are often the first non-breastmilk food given to infants, followed by high carbohydrate based, soft, weaning foods (e.g., porridge or gruel)[36]. Usually by 2 years, infants are eating the same foods as adults.

The Yao people in Malawi, as well as the Chagga, Wagogo, and Haya in Tanzania traditionally give prelacteal feeds (artificial feeds or drinks given to an infant before breastfeeding is initiated) to infants with the intention of preventing certain childhood illnesses[227]. The Tumbuka in rural northern Malawi boil crushed leaves in water and introduce it as the first non-breast milk substance given to infants. It is believed to protect infants from the illness 'moto,' which presents symptoms such as coughing, difficulty breathing, and low body weight. The Wagogo in central Tanzania give infants sugar or salt water to infants immediately after birth. In reports from the late 1990s, the Datoga pastoralists in northern Tanzania would sometimes feed infants a mixture of water and hot ash[228]. Among the Wagogo, traditional beliefs include maternal illness as indicating the mother's milk would become bad[229]. Similarly, milk that is expressed too infrequently is thought to also be bad and is discarded. Subsequent pregnancies are perceived to alter the mother's body in such a way that makes the milk indigestible and liable to cause diarrhea to nursing offspring.

Types of complementary foods introduced to offspring are often dependent on adult diets. For example, pastoralist tribes like the Datoga largely give cow's milk as a supplementary feed whereas tribes like the Wagogo and Tumbuka give cereal-based gruels. The 'weaning food availability' hypothesis predicts that introduction of complementary foods and breastfeeding cessation will occur earlier in populations with regular access to easily

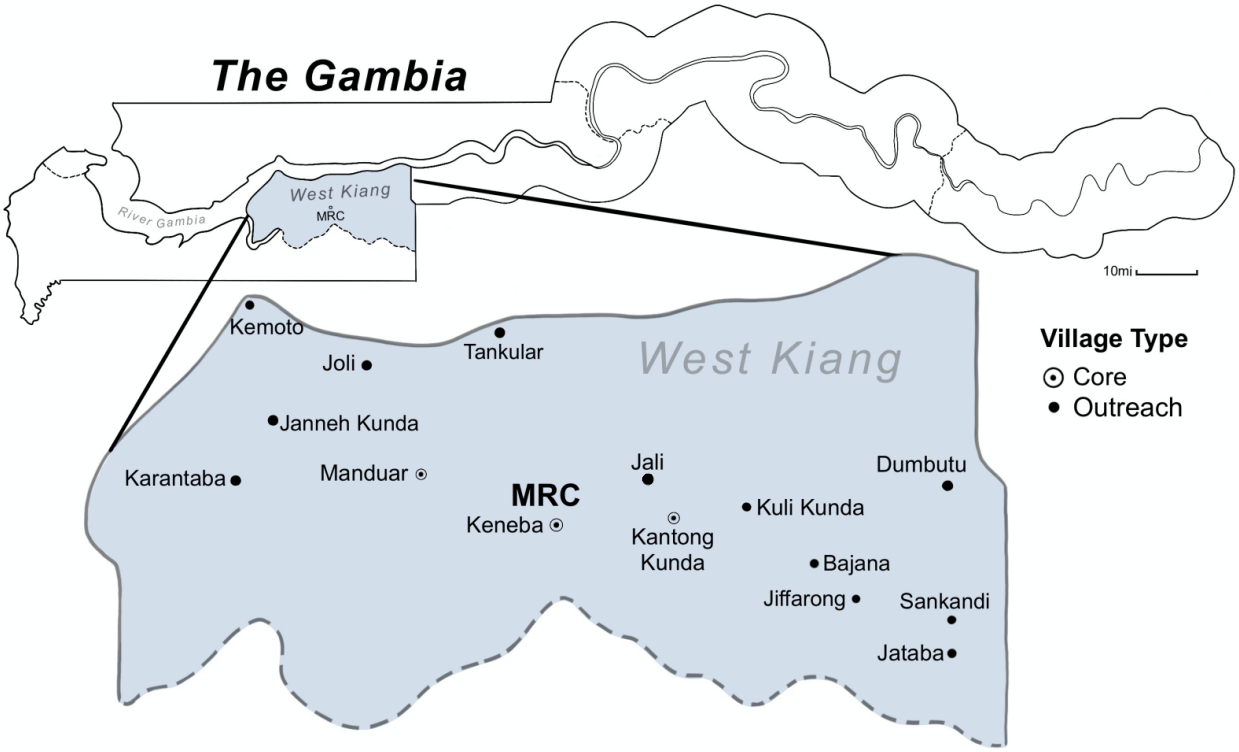
digestible, nutrient-dense complementary foods that pose minimum health risk to weaning offspring[36]. In some paleodemographic literature, it is assumed that in ancient populations that adopted pastoral and agricultural subsistence patterns, the availability of starchy weaning foods and non-human milks lead to earlier introduction of non-breast milk foods. This supported the idea that early weaning contributed to reduced inter-birth intervals and increased fertility, but also potentially increased infant mortality[230–232]. Other evidence, however, suggests that introduction of complementary foods was delayed in agricultural populations relative to those less dependent on agriculture, and introduction of solid foods was delayed in pastoral groups compared to those less dependent on herding[36]. Overall, the weaning food availability hypothesis is largely rejected in the literature. An analysis of the Human Relations Area Files (HRAF) revealed differences in types and timing of supplementation between agriculturalists, pastoralists, and hunter-gatherers[36]. Hunter-gatherers introduce liquids very early and completely wean their infants later than other societies. Liquid supplementation in agricultural groups and solid food supplementation in pastoralists were relatively delayed compared to other subsistence groups. However, in general, subsistence type played little role in the cessation of breastfeeding. Instead, breastfeeding patterns are more likely to be influenced by resource availability[233] and mother's work schedule[144] regardless of the subsistence base of the population.

SUMMARY AND HERO-G STUDY INTRODUCTION

The dynamic nature of lactation strategies and milk composition allow both mother and infant to adaptively respond physiologically and behaviorally to a variety of ecological circumstances. Information regarding these circumstances, based on both maternal exposures and current infant environment, is conveyed to infants through nutrients and bioactive factors in maternal milk[214]. While the relationships between human milk macronutrients, bioactives, and offspring development have been examined in the literature, the specific connections between HMO and HMGP content and infant growth phenotypes has yet to be comprehensively characterized. This information is particularly understudied in populations where undernutrition is pervasive, although a comprehensive understanding of the influence of early life diet on health and growth outcomes is of particular importance in rural, low-income populations such as those in the West Kiang region of The Gambia in West Africa (**Figure 1.5**), where growth faltering during childhood is common and is linked with high rates of morbidity and mortality[234,235]. Here, physiology is influenced by marked seasonality, with a long dry "harvest" season (November-June), and a short wet "hungry" season

(July-October)[236], associated with fluctuations in rates of infections, intensive agricultural workloads, and food insecurity. Pregnant and lactating women are not exempt from this seasonal hardship, resulting in seasonal changes in gestational weight gains, birth outcomes and infant growth[237–239]. Breastfeeding is nearly universal in The Gambia, and the only sustainable option for many.

Figure 1.5. Map of The Gambia (West Kiang region shaded in gray)



MRC: Medical Research Council Keneba field station
Note: There are a total of 36 villages currently registered within the West Kiang Demographic Surveillance System. Only the villages included in the present analysis are depicted on the map.

The Gambia country profile

The Gambia is the smallest country in mainland Africa, situated on the Atlantic coast and bounded by Senegal. The population is estimated around 2.4 million people[240,241]. The country is divided into 6 regions (**Figure 1.5**) and contains a wealth of landscapes, including coastal, marine, savanna, and wetland habitats[242–245]. The West Kiang region of the country is comprised of 36 villages. The 'core' villages (Keneba, Manduar, Kantong Kunda, and for a limited time Jali) have been involved in longitudinal demographic and health surveillance since 1950; provision

of healthcare and implementation of research studies began to extend beyond the core villages to what are referred to as 'outreach' villages[246]. Villages in this area are divided into compounds. Polygynous marriages, associated with the predominant Islam religion in the country, are normative in The Gambia, and households generally live in compounds with all family members[247]. On average, each compound is inhabited by 16 people, but this can range from 1-170[246].

There are numerous ethnic groups in The Gambia, including (but not limited to) the Fula, Mandinka, Jola, Wolof, and Serahuli people. In the West Kiang region, the Mandinka ethnicity represents 79.9% of the population, followed by Fula (16.2%), Jola (2.4%), and other (1.3%). The Mandinka and Wolof are traditionally sedentary crop farmers; the Fula are traditionally considered pastoralists, though many have turned to agropastoralism[248]. Over 50% of the Gambian adult population has received no formal education, and this proportion is higher in more rural areas of the country (such as in the West Kiang region)[246].

The country is known, in part, for its tourism industry and rice production. Growing crops and raising livestock are central to the livelihoods in populations across The Gambia[249–252]. Use of livestock such as cattle, goats, sheep, and poultry are common. Cattle are considered the most important to livelihoods, whereas goats and sheep are important sources of income[248,253,254]. Gambian agriculture consists of crops such as rice, millet, groundnuts, and maize[255,256]. The agriculture sector is the leading component of the Gambian economy, employing 75% of the country's labor force and contributing nearly 18% of the national Gross Domestic Product in 2019[241,244,257]. The Multiple Indicator Cluster Survey (MICS) in 2018 reports that nearly 91% of survey respondents from rural areas (N=19,191) of the country own livestock[258]. Women play a particularly prominent role in the agriculture sector in The Gambia. More than 90% of agricultural production is done by small-scale producers, around 70% of which are women[242,245,259,260].

Both income and dietary patterns fluctuate in accordance with the annual farming calendar in The Gambia. During the annual rainy season (July-October), the country receives an average of 40 inches of rain. Crop growth predominantly occurs during the wet season whereas crop harvesting takes place at the end of the wet season and into the start of the dry season. As such, the wet season is characterized by reduced food availability, increased maternal agricultural workload, and increased morbidity and mortality. Food stores improve, physical labor related to subsistence practice is lessened, and morbidity prevalence is reduced during the dry season.

The current under-five mortality rate in The Gambia is 57 per 1,000 live births, and infant mortality occurs in 41 out of every 1000 live births[258]. More than 25% of children under 5 years of age in The Gambia are affected by

chronic undernutrition, which can result in growth stunting and other health-related complications. Prevalence of undernutrition has increased in recent years, largely impacting rural areas of the country. Of the deaths occurring during the first 5 years of life, 33% were caused by malaria, 17% by pneumonia, 12% by diarrheal diseases, and 10% by non-communicable diseases[258]. Stunting affects 19% of those under five in The Gambia, and 6.2% are affected by wasting[261].

There has been a strong push in recent decades to improve the nutritional status of people in The Gambia. The Medical Research Council (MRC) Unit, The Gambia has been conducting research in nutrition and related subjects in The Gambia for over 60 years. This work is aimed at reducing the burden illness and mortality in low- and middle-income countries (LMICs), and improving health practices and policies that maximize the health impact of their research. The MRC Keneba field station clinic provides general health care to all citizens from the West Kiang region who seek medical treatment[246].

In addition to the research conducted through the MRC, other efforts are in effect to improve nutritional conditions, including the National Nutrition Agency (NaNA). Established in 2000, NaNA is involved in the implementation and coordination of the National Nutrition Policy in The Gambia, which involves efforts such as increasing visibility, expanding the funding base, and implementing nutrition programs in various communities[262]. These programs aim for goals such as empowering communities to improve maternal, infant, and young child nutrition, reducing or eliminating micronutrient deficiencies, and promoting breastfeeding.

Study population

I use data collected as a part of the Hormonal and Epigenetic Regulators of Growth (HERO-G) study (2013-2018 active data collection), which was designed to investigate intrauterine and postnatal growth patterns in rural Gambian infants (N=238) using epigenetic, endocrine, and metabolic analyses. The HERO-G study was conducted in the West Kiang region of The Gambia. Full details of the HERO-G study can be found in the published study protocol[263].

For the purposes of this dissertation, a subsample of 194 mother-infant pairs (herein referred to as the 'HERO-G subsample') was selected from the larger HERO-G study based on completeness of collected data over the first 12 months of life. Specifically, mother-infant pairs with no available infant feeding data or large gaps in dietary reports (>1 month without dietary reports) (N=44) during the first 6 months of life were excluded from this analysis. Inclusion

and exclusion criteria are described in detail in subsequent Chapters. Baseline characteristics of the full HERO-G cohort and the HERO-G subsample are described in **Table 1.1**.

Table 1.1. Full HERO-G cohort (N=238) and HERO-G subsample (N=194) maternal and infant baseline characteristics

Variable	HERO-G Cohort (N=238)	HERO-G Subsample (N=194)
Maternal age, years (SD)	31.0 (±6.9)	32.0 (±6.9)
Parity, N (%)		
Primiparous	29 (12.2)	18 (9.3)
Multiparous	209 (87.8)	176 (90.7)
Infant season of birth, N (%)		
Wet season (Jul-Oct)	84 (35.3)	64 (33.0)
Dry season (Nov-Jun)	154 (64.7)	130 (67.0)
Infant sex, N (%)		
Male	128 (53.8)	103 (53.1)
Female	110 (46.2)	91 (46.9)

Details regarding village of residence and their distances from the MRC Keneba field station clinic for the full HERO-G cohort and the HERO-G subsample are presented in **Table 1.2**. Villages were divided into "core" or "outreach" categories depending on distance from the MRC Keneba field station clinic and participation in the core research program ("core" = Kantong Kunda, Keneba, and Manduar; "outreach" = Bajana, Dumbuto, Jali, Janneh Kunda, Jattaba, Jiffarong, Joli, Karantaba, Kemoto, Kuli Kunda, Mandina, Sandeng, Sankandi, Tankular). This categorization has been used elsewhere in analyses of infant outcomes in relationship to early life diet[264]. A total of 64 mother-infant pairs were from 'core' villages and 130 from 'outreach' villages. The HERO-G study was approved by the joint Gambian Government/MRC Unit The Gambia Ethics Committee (Project No. SCC1313v3), and the University of Colorado Institutional Review Board (Protocol No. 13-0441). Written informed consent was obtained from all participants before enrollment.

Table 1.2. Full HERO-G cohort (N=238) and HERO-G subsample (N=194) village of residence and distance to MRC clinic

Village name	HERO-G Cohort (N=238)	HERO-G Subsample (N=194)	Village type*	Distance to MRC clinic (km)
Bajana	10 (4.2)	8 (4.1)	Outreach	15.1
Dumbuto	1 (0.4)	1 (0.5)	Outreach	27.6
Jali	12 (5.0)	9 (4.6)	Outreach	5.7
Janneh Kunda	15 (6.3)	14 (7.2)	Outreach	20.5
Jattaba	7 (2.9)	4 (2.1)	Outreach	24.2
Jiffarong	28 (11.8)	24 (12.4)	Outreach	17.5
Joli	10 (4.2)	7 (3.6)	Outreach	13.6
Kantong Kunda	13 (5.5)	13 (6.7)	Core	13.4
Karantaba	8 (3.4)	6 (3.1)	Outreach	16.8
Kemoto	5 (2.1)	4 (2.1)	Outreach	21
Keneba	47 (19.7)	41 (21.1)	Core	0
Kuli Kunda	22 (9.2)	18 (9.3)	Outreach	10
Mandina	1 (0.4)	0 (0.0)	Outreach	56.2
Manduar	16 (6.7)	10 (5.2)	Core	7.5
Nyorro Jattaba	18 (7.6)	15 (7.7)	Outreach	24.2
Sandeng	1 (0.4)	1 (0.5)	Outreach	26
Sankandi	7 (2.9)	5 (2.6)	Outreach	22.1
Tankular	17 (7.1)	14 (7.2)	Outreach	10.8

Data are presented as N (%).

*Village Type: villages were divided into "core" or "outreach" categories depending on distance from the MRC Keneba field station and clinic and participation in the core research program ("core" = Kantong Kunda, Keneba, and Manduar; "outreach" = Bajana, Dumbuto, Jali, Janneh Kunda, Jattaba, Jiffarong, Joli, Karantaba, Kemoto, Kuli Kunda, Mandina, Sandeng, Sankandi, Tankular). This categorization has been used elsewhere[264].

HERO-G METHODS

Sample collection

Sample and data collection conducted in the HERO-G study and incorporated into the present analyses are summarized in **Table 1.3** Specific sample and data collection methodologies are described in greater detail in their respective Chapters. Below, I briefly outline the pre- and postnatal data collected as a part of the HERO-G study and specify the data incorporated into the dissertation analysis and, where appropriate, the inclusion/exclusion criteria and justifications used to select certain subsets of data.

Prenatal

Socioeconomic questionnaires were administered during the 'booking' visit (the first clinic visit after pregnancy confirmation – variable gestational age) for the HERO-G study. Mothers were asked to provide information

regarding sociodemographic variables (maternal education attainment), household characteristics (number of persons per room within the household, material of dwelling walls and floor), and durable assets (livestock ownership, possession of a cart).

Maternal anthropometric measurements (weight, height, lower leg length, mid-upper arm circumference (MUAC) and triceps skinfold thickness (TSF)) were collected at 20, 28, and 36 weeks of gestation (*Note:* HERO-G study participants who were < 20 weeks pregnant were seen at the booking visit and then at 20, 28 and 36 weeks' gestation. Participants who were > 20 weeks pregnant were seen at booking, 28 and 36 weeks only). The primary maternal anthropometric measurement of interest in this dissertation is MUAC. At the scheduled prenatal clinic visits, a HERO-G study midwife measured maternal MUAC using flexible measuring tape (Seca 212) to the nearest 0.1mm[263]. Undernutrition was classified in this dissertation as a MUAC value of < 23 cm[265]. MUAC measurements at 36 weeks (third trimester) specifically are incorporated into this dissertation as a proxy of general maternal condition/nutritional status during pregnancy, an approach used elsewhere in the existing literature[266,267].

Postnatal

Mothers and infants traditionally stay home together to rest and recover for a week after birth, after which a naming ceremony is held. Starting at one week of age, dietary questionnaires regarding infant feeding were administered to mothers by trained field workers every 10 days until 12 months of infant age. Mothers or caregivers were asked to recall infant feeding practices (breastfeeding status, if non-breast milk foods were given, specification of food type, etc.) in the previous 10 days. Mother-infant pairs with no available infant feeding data and those missing infant feeding data from three consecutive visits (the equivalent of one month) during the first 6 months of life were excluded from the analyses for this dissertation. Questionnaire responses documented across the entire data collection period were incorporated and analyzed for individuals missing infant feeding data from < 3 consecutive visits during that same time period.

'Scheduled clinic visits' refer to the time points in which mother-infant pairs were scheduled to be seen at the MRC clinic for data and sample collections. The scheduled clinic visits were conducted at 3, 6, 9, 12, 18, and 24 months at the MRC field station and biological samples and other data were collected from both mother and infant at these time points. I describe the methodology for the scheduled clinic visit samples and data collection below.

First, mothers hand-expressed milk for sample collection at each of the scheduled clinic visit time points. Only the maternal milk samples collected over the first year of life were incorporated into my assessments of milk composition because the first 12 months are the primary focus of this dissertation.

Infant anthropometric measurements (weight, length, head circumference, MUAC, lower leg length, and TSF) were collected during all scheduled clinic visits. These measurements were also collected at home visits every other day until 12 months of age; however, the focus here is on the measurements taken specifically at the scheduled clinic visits (not the alternate day home visits) at 3, 6, 9, and 12 months of age. Infant growth measurements were indexed using height-for-age (HAZ), weight-for-age (WAZ), and weight-for-height (WHZ) Z-scores according to the WHO Child Growth Standards[84] by using WHO Anthro program (Version 3.2.2).

When possible, mothers used a stool collection kit to collect an infant fecal sample prior to the scheduled clinic visits. Stool samples were stored on ice in a cooler box delivered to the mother's home the day of collection and brought with them to their infant's clinic visits.

Infant morbidities were diagnosed and recorded by clinicians at the MRC Keneba field station at the scheduled clinic visits and at 'unscheduled clinic visits' (caregivers sought and were provided MRC clinical evaluation and treatment for infant morbidity as needed). This dissertation focuses its primary analysis on cumulative morbidity occurrences reported at 3, 6, 9, and 12 months age.

Table 1.3. Pre- and postnatal data collection for study aspects pertinent to this dissertation.

Study Stage	SEP Survey	Maternal Anthrop.	Infant Anthrop.	Infant DQ	Maternal Milk	Infant Stool	Infant Health Eval.
Booking[a]	X	X					
20wks gestation[b]		X					
28wks gestation		X					
36wks gestation		X					
Delivery & 1wk			X				
Alternate days between 1wk-12mo			X				
Every 10 days between 1wk-12mo				X			
3mo			X		X	X	X
6mo			X		X	X	X
9mo			X		X	X	X
12mo			X		X	X	X
18mo			X		X	X	X
24mo			X		X	X	X
Other: Unscheduled/As Needed[c]							X

[a]'Booking' refers to the first clinic visit after pregnancy confirmation – variable gestational age; [b]HERO-G study participants who were < 20 weeks' gestation were seen at 'booking', and then at 20, 28 and 36 weeks' gestation. If women were > 20 weeks pregnant, they were seen at booking, 28 and 36 weeks only; [c]Unscheduled/As needed refers to caregivers sought and were provided MRC clinical evaluation and treatment for infant morbidity as needed; SEP Survey: Socioeconomic position survey; Maternal Anthrop.: Maternal Anthropometry Measurements (weight, height, lower leg length, mid-upper arm circumference (MUAC) and triceps skinfold thickness (TSF)); Infant Anthropometry Measurements (weight, length, head circumference, MUAC, lower leg length, and TSF); Infant DQ: Infant Dietary Questionnaire (exclusive breastfeeding status, timing, type, and frequency of non-breast milk food use); Infant Stool: Where possible, infant fecal samples were collected at home by caregiver on the day of clinic visit.; Infant Health Eval: Infant Health Evaluation (Infant morbidities were diagnosed and recorded by clinicians at the MRC Keneba field station)

STATISTICAL ANALYSES

Statistical analyses specific to each component of the present analysis are described in their respective Chapter. Briefly, G*Power 3.1 was used to determine statistical power of sample sizes. The associations found in the statistical models were summarized using the beta regression coefficients and 95% confidence intervals (CI). Non-parametric tests were used where data distribution was non-normal. I constructed Normal Quantile Plots and visually assessed normality. Normality was then assessed using a Goodness-of-Fit Test. Multiple linear regression models were constructed and descriptive statistics were calculated for all variables included in the statistical models. The level of statistical significance was set to $P < 0.05$ for all evaluations. All statistical analyses were conducted using JMP Pro

15.0 statistical software (©2019 SAS Institute, Inc.). Full methodological details are described in the Appendix. HERO-G study protocols are published and available elsewhere[268].

CHAPTER DESCRIPTIONS

In Chapter 1, the context and background of this dissertation were introduced. The overall aim was identified and the significance of this work was described.

In Chapter 2, I will provide biocultural background on infant feeding practices in The Gambia and the factors that may influence decisions to introduce non-breast milk foods. I will then characterize the breastfeeding and complementary feeding practices in the HERO-G subsample based on analysis of dietary questionnaires collected over the first 12 months of life.

In Chapter 3, I will assess rural Gambian maternal milk composition by measuring the macronutrient concentrations and relative abundance of HMO and HMGP across the first 12 months of lactation. A full characterization of all milk constituents is beyond the scope of this thesis. In these analyses, I focus on milk macronutrients (fat, protein, lactose, and true protein), HMO classes (fucosylated, sialylated, undecorated, and sialylated and fucosylated) and immune proteins (lactoferrin and immunoglobulin A).

In Chapter 4, I will provide an overview of infant immune system function and its relationship to early life diet across the first year of life. I will then assess the effects of variation in the duration of exclusive breastfeeding on infant morbidity occurrence.

In Chapter 5, I will investigate how breastfeeding practices and maternal milk composition impact infant health (defined by morbidity occurrence), growth outcomes (WHZ, HAZ, WAZ), and intestinal ecology as defined by gut pH, in the context of seasonality.

In Chapter 6, I will synthesize the results from all Chapters and offer suggestions for further research that may benefit unanswered questions.

CHAPTER 2. BREASTFEEDING & COMPLEMENTARY FEEDING PRACTICES

INTRODUCTION

Suboptimal complementary feeding practices can result in a cycle of undernutrition and morbidity[155,168,269–272]. The World Health Organization (WHO) recommends exclusive breastfeeding until 6 months of age, followed by introduction of safe and nutritionally adequate foods in addition to continued breastfeeding until 24 months of age[273]. This duration has been identified as a key intervention for reducing mortality during childhood. However, a large proportion of infants begin consuming non-breast milk foods much earlier[155,274–277]. In The Gambia in West Africa, earlier introduction of non-breast milk foods may relate to maternal agricultural workload in this particular subsistence economy, where the physical activity level of mothers has been reported to return to pre-pregnancy levels after the first month post-partum[278]. This increase in workload may relate to a decision to begin introducing complementary foods earlier.

In this Chapter, I characterize infant breastfeeding and complementary feeding practices in a subsample (N=194) from the larger HERO-G cohort. This includes maternal reports of exclusive breastfeeding duration (provision of maternal milk only; no other liquids or solids are given – not even water) and type of complementary foods introduced as the first non-breast milk foods into the infant diet. I then assess potential predictors of exclusive breastfeeding duration and discuss all results in the context of environmental and sociodemographic characteristics in this population.

BACKGROUND

Infant feeding practices in The Gambia

More than 95% of infants in The Gambia receive maternal milk throughout the first year of life and nearly half continue to breastfeed until 2 years of age[258,264,279]. Nonetheless, prior studies in The Gambia report that more than 25% of children under 5 years of age, an increase over recent years, are affected by chronic undernutrition[262,269]. Several studies have documented average cessation of exclusive breastfeeding in The Gambia as falling between 3 to 6 months of age[225,258,274,280–282]. There are some differences in infant feeding practices between urban and rural areas of The Gambia. The 2018 UNICEF Multiple Indicator Cluster Survey reports that 58.2% of Gambian infants from rural areas (N=343) were exclusively breastfed to 5 months of age whereas 50.9% were exclusively breastfed to the

same age in urban areas[258]. The median duration of any breastfeeding was 21.4 months for those from rural areas of the country[258] and the median duration of exclusive breastfeeding was 3.3 months (N=1,412)[258].

Infant feeding practices and access to nutritious foods are associated with household socioeconomic position (SEP), which can add complexity to infant feeding decisions. In The Gambia, children from rural communities had significantly higher odds of not meeting the requirement for minimum acceptable diet (a core indicator for assessing infant and young child feeding practices developed by the WHO; includes minimum dietary diversity and minimum meal frequency) compared with their urban counterparts[269,283,284]. This may relate to a number of factors, which Issaka et al. (2017) attribute to variables such as general household poverty[285].

Though there are some differences between urban and rural populations in The Gambia, the available literature suggests that human milk is an important component of Gambian infant feeding patterns even after cessation of exclusive breastfeeding[286]. Here, I focus on studies from rural Gambian populations, emphasizing those from the West Kiang Region.

Local complementary foods

While many Gambian mothers breastfeed their infants for 18-24 months, the percentage of rural infants breastfed within one hour of birth is relatively low (44.1%)[258] and sometimes delayed at least one day after delivery[223]. In one study, around 8% of cases used wet nurses to feed the infant for the first few days of life as opposed to the infant's mother in order to avoid feeding the baby colostrum[226]. In other cases in The Gambia, infants who do not receive colostrum are instead given warm water with or without sugar (and occasionally salt), cow's milk, or formula, until the mother begins to produce mature milk[222,226]. The WHO states that prelacteal feeds are dangerous for a number of reasons. Prelacteal feeds, such as those used to replace colostrum, have been shown to increase an infant's risk of developing infections such as diarrhea, septicemia, meningitis, and various allergies and atopies[287].

Traditional local weaning foods in rural Gambia include cereal dishes and other staples, such as rice (local name, 'mani'), millet ('sanyo', 'suno'), and maize ('tubanyo'), which are among the staple crops grown in this region. These foods are generally prepared as thick ('mono') or thin/watery ('jidiyo') porridges for infants. Meat, fish, and animal milk are used less frequently as complementary foods[280,281,288]. Nutritional composition of raw ingredients and dietary information for recipes of locally prepared dishes (e.g., weaning porridges) have been reported by previous studies[289–292].

The annual cropping patterns determine food supply throughout the year, and thus the availability of certain ingredients for complementary foods. Cereal crops are generally harvested between September and December in rural Gambia (most observations cited were made in Keneba, a remote subsistence farming village in the West Kiang Region), resulting in particularly plentiful food supplies in November and December[293]. Food shortages often occur in July and August. For example, the availability of groundnuts, the chief cash crop in the country and a primary ingredient of 'tiakere churo' (rice and groundnut porridge), decreases in August/September when the new crop has been planted but is not yet harvestable[293]. When food is scarce in the wet ("hungry") season, 'jidiyo,' a watery/thin gruel made with powder or any pounded grain, may be preferentially used over a thicker, 'mono,' porridge[293].

Infant feeding decisions

In rural Gambia, infant feeding patterns have been shown to depend on overall maternal workload, location of work/distance of work from home, ease of work interruption (ability to return home during the day to feed infant), and the extent to which childcare can be shared with others[294]. Women in rural Gambia are largely responsible for childcare, farm work, and routine household chores; men are responsible for cattle-breeding (with hired herdsmen – who are predominantly male – responsible for milking and general animal care) and do not generally help with the upbringing of children[295–297]. Women in the West Kiang region of The Gambia work for long periods (around 15 hours/day) in agricultural fields, conducting physically intensive tasks such as land preparation, planting, weeding, watering, harvesting, and transporting[298]. Agricultural workload increases in the wet ("hungry") season. Rural Gambian women report that engaging in laborious work under harsh environmental conditions sometimes comes at the expense of the child's well-being, including reduction of time allocated towards child health and nutritional needs[295]. In The Gambia, an increase in subsistence activities during the wet season leads to mothers separating from their infants from morning to evening to work[299,300].

Polygynous marriages, associated with the predominant Islam religion in the country, are normative in The Gambia, and households generally live in compounds with all family members[247]. Because the increase in subsistence activities during the wet season can lead to mothers leaving home to work in the fields, infants are often separated from their mothers from morning to evening to work during periods of heavy agricultural workloads[299,300]. During these times, mothers often rely on other children or an elderly person within their compound to care for their own child[226,295,309,310]; this allows for shared caregiving responsibility by individuals other than the mother[36,39]. Some

Gambian mothers report that grandmothers have an especially influential role on early introduction of complementary foods[223].

Fouts et al. (2005) and Ghosh et al. (2006) report that the amount of physical effort required to carry a child to the field is a reason to introduce non-breast milk foods at earlier ages[144,311]. Data from the ENID Bioactives study (Early Nutrition and Immune Development; active data collection 2010-15) show that during the dry season, the majority of mothers brought infants, as young as 1 week of age, with them while working in the fields. During the wet season, around 50% of mothers left infants less than 6 months old at home, suggesting a reduction in maternal time investment in offspring during periods of intensive agricultural labor. Few mothers (21.3%) reported returning home during the work day to feed their infants and only 10.9% reported expressing breast milk for infant to consume at home while she is away working[312]. When infants receive care from others during the work day, earlier cessation of exclusive breastfeeding may be a necessary alteration to infant feeding[313,314]. In older research (>40 years ago), reports indicate that it was common practice in The Gambia for infant foods to be prepared in large quantities in the morning, sufficient enough for several meals throughout the day and so it is available if another caregiver is responsible for feeding the infant while the mother is away[278302]. It is important to note that this may be out of date. This form of food preparation is convenient and efficient, but has an associated downside of high levels of pathogenic bacterial contamination due to extended and poor storage conditions[301–307]. After preparation, common local complementary foods are stored at ambient temperatures, which allows the child to be fed on demand[302]. Additionally, these traditional complementary foods and some of their commonly used ingredients have been shown to contain significant microbial contamination, including high abundance of pathogens such as *Staphylococcus aureus* and *Escherichia coli (E. coli),* which are associated with gastrointestinal infection[306–308]. In their research, Barrell and Rowland (1979) found that foods that were not consumed fresh became bacterially contaminated after 8 hours in ambient temperature. In addition, foods prepared in the wet season contained higher levels of potential pathogens compared to foods prepared during the dry season, presumably because the environmental conditions during the wet season are very hospitable for bacterial growth[302]. In The Gambia and other regions of the world, some gruels are prepared using contaminated water that contains potentially pathogenic coliform bacteria from both animal and human sources[315], though this too is out of date research.

RESEARCH AIMS

Aims

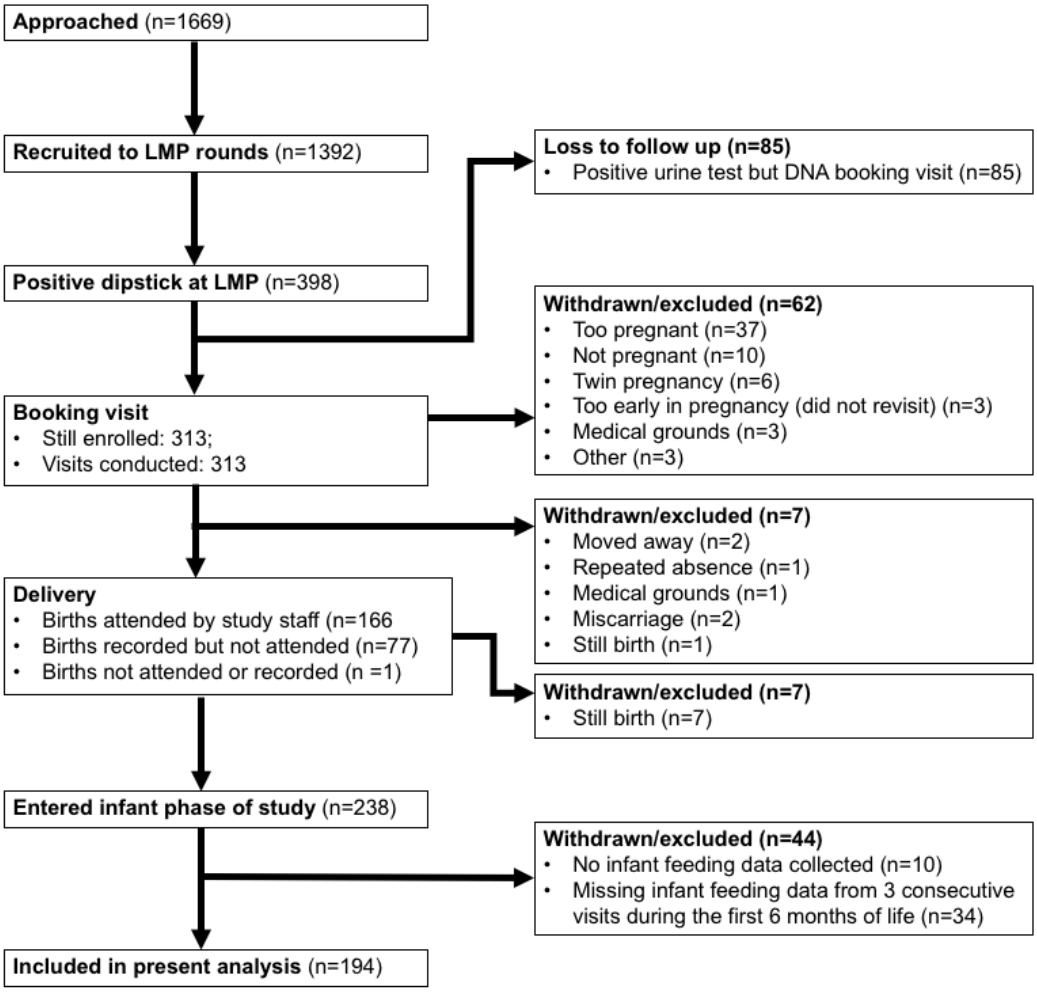
In this Chapter, I assess infant breastfeeding practices and the timing of introduction of the first non-breast milk foods in the HERO-G (Hormonal and Epigenetic Regulators of Growth) cohort in order to characterize infant exclusive breastfeeding duration and introduction of specific local weaning foods. In the West Kiang Region of The Gambia, where the HERO-G study was carried out, subsistence farming is central to the livelihoods and diets of the communities, and women shoulder much of the agricultural workload. Understanding infant feeding practices in this rural environment provides contextual information regarding the significance of first foods infants receive as they relate to environmental conditions, and in turn, subsequent health and growth outcomes.

METHODS

Subsample

I utilize data from a subsample (N=194) of mother-infant pairs from the larger HERO-G cohort, who were included in the analysis based on completeness of collected data over the first 12 months of life. Specifically, mother-infant pairs with no available infant feeding data (N=10), and those missing infant feeding data from three consecutive visits (the equivalent of one month – see questionnaire methodology below) during the first 6 months of life (N=34) were excluded from this analysis (**Figure 2.1**). Instances of no available infant feeding data were attributable to unavailability of mother at the time of questionnaire distribution, either related to maternal travel or undocumented reasons. In many cases, missing infant feeding data from > 3 consecutive reports of infant feeding practices occurred if mothers were traveling or working and thus unavailable to provide responses to the questionnaire. To determine the statistical power of a sample size of 194 in examining associations between exclusive breastfeeding (EBF) duration and maternal, infant, and environmental factors using multiple linear regression (F-test), a post-hoc power analysis was conducted using G*Power 3.1[316] was conducted. With a medium effect size (f^2=0.15) and a significance level of $\alpha = 0.05$, a sample size of 194 has a power (1-ß err prob) of 0.99.

Figure 2.1. Flow diagram of included and excluded HERO-G participants in the HERO-G subsample



Infant feeding practices

Dietary questionnaires regarding infant feeding were administered to mothers by trained field workers every 10 days starting at one week of infant age (mothers and infants traditionally stay home together to rest and recover for a week after birth, after which a naming ceremony is held) until 12 months of infant age. Mothers or caregivers were asked to recall infant feeding practices in the previous 10 days. Questions included those such as infant breastfeeding status, if non-breast milk foods were given, the frequency of intake of those foods or liquids, and specification of food type (e.g., water, tea, cow's milk, watery or thick gruel, etc.). The full dietary questionnaire is detailed in **Table 2.1.**

Table 2.1 Infant feeding questionnaire

1. Infant feeding			Y/N
1.1	Are you currently breast feeding your infant?	No [0]	Yes [1]
1.2	In the past 10 days, have you given your infant anything other than breast milk?	No [0]	Yes [1]
1.3	If no, the questions are completed. If yes, please proceed to Question 2.	No [0]	Yes [1]
1.4	What other foods/Drinks have been given, in the past 10 days	No [0]	Yes [1]

2. Drinks (Frequency: 1=Once, 2=>Once, 3=Most days, 4=Never)

		Y/N		Frequency	Comment
2.1	Water	No [0]	Yes [1]		Freetext
2.2	Cow's milk	No [0]	Yes [1]		Freetext
2.3	Tinned milk	No [0]	Yes [1]		Freetext
2.4	Powdered milk	No [0]	Yes [1]		Freetext
2.5	Other (specify)	Freetext			

3. Semi-solids (Frequency: 1=Once, 2=>Once, 3=Most days, 4=Never)

3.1	Types	Mono[a]=1	Jidiyo[b]=2		
		Y/N		Frequency	Comment
3.2	Sayno	No [0]	Yes [1]		Freetext
3.3	Mani	No [0]	Yes [1]		Freetext
3.4	Tubanyo	No [0]	Yes [1]		Freetext
3.5	Dukula	No [0]	Yes [1]		Freetext
3.6	Sunno	No [0]	Yes [1]		Freetext
3.7	Tiakere churo	No [0]	Yes [1]		Freetext
3.8	Other (specify)	Freetext			

4. Solids (Mother to list) (Frequency: 1=Once, 2=>Once, 3=Most days, 4=Never)

	Food name	Frequency	Comment
4.1	Freetext		Freetext
4.2	Other (specify)		Freetext

5. High protein food (Mother to list) Frequency: 1=once,2=>once,3=Most days,4=never

	Food name	Frequency	Comment
5.1	Freetext		Freetext
5.2	Other (specify)		Freetext

[a]Mono: thick gruel; [b]Jidiyo: watery/thin gruel

Socioeconomic position

Fieldworkers administered a socioeconomic questionnaire during the booking visit for the HERO-G study (see HERO-G Project Protocol Flow Chart in Chapter 1). Mothers were asked to provide information regarding sociodemographic variables (maternal education attainment), household characteristics (crowding index [number of persons per room within a dwelling], material of dwelling walls and floor), and durable assets (livestock ownership, possession of a cart). Details of the full socioeconomic questionnaire are described in **Table 2.2.** Other economic

indicators commonly used in assessments of socioeconomic position (SEP), such as occupation, income, consumption expenditure, water source, ownership of a bicycle or vehicle, ownership of a radio, and/or access to electricity, were not documented as a part of the HERO-G study. This dissertation focuses only on the responses collected from mothers included in subsample (N=194) from the larger HERO-G study (N=238).

Table 2.2. HERO-G socioeconomic questionnaire administered at the booking visit

Sociodemographic

	Item	Units and/or Ranges
1.	*Maternal Education*	
1.1	Which is the highest grade you completed in English school?	Range: 1 - 12; Tertiary [99]; Don't know [888]; N/A or still in school [999]; Never went to school [0]
1.2	Are you still in education?	No [0]; Yes [1]; N/A [9]

Household Characteristics

	Item	Units and/or Ranges
2.	*Household size*	
2.1	How many rooms do you live and sleep in?	Range: 1-20; Unknown [88]
2.2	Including yourself, how many people live in these rooms?	Range: 1-20; Unknown [88]
3.	*Housing Materials*	
3.1	What are the walls of your house made of?	Mud [1]; Cement [2]; Other [3]; Unknown [8]
3.1a	If Other, specify:	Freetext
3.2	What is the floor of your house made of?	Mud [1]; Cement [2]; Other [3]; Unknown [8]
3.2a	If Other, specify:	Freetext

Durable Assets

	Item	Units and/or Ranges
4.	*Livestock Ownership*	
4.1	How many sheep do you own?	Range: 0 - 200; Unknown [88]
4.2	How many goats do you own?	Range: 0 - 200; Unknown [88]
4.3	How many cows do you own?	Range: 0 - 200; Unknown [88]
5.	*Possessions*	
5.1	Do you own a cart?	No [0]; Yes [1]; Unknown [8]

Employment and household income are variable and difficult to measure in settings such as rural Gambia. For example, broad occupation information may not accurately capture the individual income for self-employed farmers in rural Gambia if it cannot take into account factors such as crop type, seasonality, and transitory market conditions[317]. Thus, asset scores are often calculated in order to measure relative wealth. Here, available data for variables documented in the above questionnaire describing sociodemographic characteristics, household characteristics, and durable assets were used to generate an asset score using Principal Component Analysis (PCA). PCA is a technique to reduce the dimensionality – the number of dimensions, features, or input variables associated in a dataset – by minimizing the number of variables needed to explain the maximum amount of variance in the dataset. First, data were cleaned and descriptive statistics were calculated to determine the distributions of participant responses. Categorical variables were re-coded in order to meet continuous variable requirements of PCA analyses. Next, JMP Pro 15.0 statistical software (©2019 SAS Institute, Inc.) was used to perform the PCA using the Multivariate Methods function. It is assumed in the literature that the first principal component is an appropriate measure of economic status[318].

Item Inclusion/Exclusion

Of the 194 mothers included in the HERO-G subsample, full questionnaire responses were available for 166 individuals. A total of 10 items were collected in the questionnaire, 4 of which were used in the present PCA. Descriptive statistics and inclusion/exclusion status are described below and detailed in **Table 2.3** for each of the 10 items collected in the questionnaire. First, of the 10 items collected, 2 were removed from the PCA due to inadequate variation: (1) present enrollment in education (1.2 in Table 2.2), where only 1 participant reported being in education at the time the questionnaire was administered); and (2) cart ownership (5.1 in Table 2.2) where only 1 participant reported owning a cart. Number of years of completed maternal education was incorporated as a continuous variable in the PCA and also categorized into one of three groups based on the response distribution for descriptive purposes: No Education (0 years), Low (1-7 years), and Medium (8-14 years) Education. These categories are based on previous studies in populations from the West Kiang Region of The Gambia[264].

Additionally, livestock ownership (cow, goat, and sheep) was removed from the PCA. The value and importance of livestock species in The Gambia have well-established links to household income[242,250,319] and studies in the West Kiang region have used cattle ownership as a single indicator of wealth (households owning < 10 cattle

considered poor and those owning > 10 cattle considered wealthy[320]). However, without data on female-only ownership, male-only ownership, or mixed ownership of livestock, confirmation that the mothers enrolled in HERO-G were the sole owner (which is unlikely because of the distinct gender differences in livestock ownership practices in the country) is not feasible. Because the potential inconsistencies in reporting based on varying definitions of ownership (for example, a mother may report owning 8 cows when the cows are owned by a co-wife's family who lives in the same compound, which therefore may not adequately represent individual household wealth) may influence the accuracy of the explanatory power of the variable, livestock ownership was excluded as a variable from further calculations. The WHO Housing and Health Guidelines (2018) defines crowding as more than 1 person per room, and severe crowding as more than 1.5 persons per room. Thus, crowding index was calculated using a ratio between number of rooms within the dwelling (2.1 in Table 2.2) and the number of persons living in the dwelling (2.2 in Table 2.2), thus combining 2 items from the questionnaire into 1 value. Crowding was coded as 0 and a non-crowded dwelling was coded as 1. Full descriptive statistics are presented below.

Table 2.3. Descriptive statistics of SEP questionnaire responses and excluded/included variables

Item	Value	Included/Excluded
1. Sociodemographic		
Maternal education attainment, N (%)		Included (treated as continuous)
No education	125 (75.3)	
Low (1-7 years)	17 (10.2)	
Medium (8-14 years)	24 (14.5)	
Present education enrollment, N (%)		Excluded (inadequate variation)
Yes	1 (0.6)	
No	40 (26.5)	
N/A	125 (75.3)	
2. Household Characteristics		
Crowding index, (people:rooms)		Included (treated as continuous)
< 1	21 (12.7)	
≥1 (crowded)	145 (87.3)	
Wall material, N (%)		Included
Mud	151 (91.0)	
Cement	15 (9.0)	
Other	0 (0.0)	
Unknown	0 (0.0)	
Floor material, N (%)		Included
Mud	55 (33.1)	
Cement	111 (66.9)	
Other	0 (0.0)	
Unknown	0 (0.0)	
3. Durable Assets		
Livestock Ownership		
Sheep, N (%)		Excluded
1	7 (3.6)	
2	6 (3.6)	
5+	3 (1.8)	
Goats, N (%)		Excluded
1	26 (15.7)	
2	20 (12.0)	
3	17 (10.2)	
4	10 (6.0)	
5+	13 (7.8)	
Cattle, N (%)		Excluded
1	5 (3.0)	
2+	5 (3.0)	
Possessions, N (%)		Excluded (inadequate variation)
Cart		
Yes	1 (0.6)	
No	165 (99.4)	

In total, three of the components had an eigenvalue of greater than one. The first principal component explained 30.2% of the variation within the data and had an eigenvalue of 1.21 and was thus considered appropriate to be used as an index. **Table 2.4** shows the eigenvectors (the weight for each eigenvalue) and the factor loadings (correlation of each item in the principal component) of the first principal component on items included in the PCA. The highest contributors to the SEP score were floor material, wall material, and household crowding. Maternal education attainment had the lowest contribution.

Table 2.4. PCA Component 1 eigenvectors and factor loadings of items included in SEP score

Item	Eigenvectors	Factor Loadings
Education attainment	0.10577	0.11629
Household Crowding	-0.41567	-0.45702
Wall Material	0.53699	0.59041
Floor Material	0.72641	0.79867

Each item was multiplied by its respective factor loading value as to account for its individual weighted contribution and then each item was summed to produce an SEP score. SEP score was used as a continuous variable in the multiple linear regression models described in the following section.

Statistical analysis

To characterize infant feeding practices, I calculated descriptive statistics from the dietary questionnaire including mean, standard deviation (SD), and ranges where appropriate. Infant feeding practice was defined by exclusive breastfeeding status at 6 months of age, based on the WHO recommended exclusive breastfeeding duration of 6 months[321]. Infants were categorized as either 'EBF <6mo' (provision of breast milk and non-breast milk foods/liquids before 6 months of age) or 'EBF ≥6mo' (provision of breast milk only until 6 months or later)[264,322].

Multiple linear regression analyses were used to investigate potential predictors of infant exclusive breastfeeding duration, including seasonality, maternal parity, and household SEP. Multicollinearity was determined using a conservative VIF > 5. The significant associations identified in the models were summarized using the beta regression coefficients and 95% confidence intervals (CI). Model effect size is reported as Cohen's f^2, where $f^2 \geq 0.02$, $f^2 \geq 0.15$, and $f^2 \geq 0.35$ represent small, medium, and large effect sizes, respectively. The level of statistical

significance was set to $P < 0.05$ for all analyses. All statistical analyses were conducted using JMP Pro 15.0 statistical software (©2019 SAS Institute, Inc.).

RESULTS

Maternal and infant characteristics

A total of 194 mother-infant pairs were included in the analysis, with a mean (±SD) maternal age of 32.0 (±6.9) years. On average, mothers had 4.5 (±2.8) children, with most mothers (N=176; 90.7%) being categorized as multiparous. Of the 194 infants, there were a total of 103 male and 91 female infants, with 145 born during the dry ("harvest") season and 49 born during the wet ("hungry") season.

Household sociodemographic characteristics

Maternal education levels were low and in line with previous findings in this population[264,286], with 75.3% of mothers having received no formal education. Over 87% of households examined in this analysis were considered crowded (people:rooms ≥1). The majority of households (N=151, 91.0%) contained wall material made from mud and the remaining households (N=15, 9.0%) had walls made of cement. Floor material was most commonly made of cement (N=111, 66.9%) followed by mud (N=55, 33.1%). Livestock ownership was mixed, with ownership of goats reported most frequently (N=86, 44.3%), followed by sheep (N=16, 9.0%) and cattle (N=10, 6.0%).

Infant feeding

The mean (±SD) age for introducing any food or liquid other than breast milk was 5.0 (±1.5) months with 59 (30.4%) infants EBF ≥6mo and 135 (69.6%) of infants EBF <6mo. The mean duration of EBF for infants categorized as EBF <6mo was 4.4 (±1.4) months and those categorized as EBF ≥6mo was 6.5 months (±0.4 months). At 1 month of age, all infants were exclusively breastfed. By 3 months of age, 5.6% of infants had been given water and 5.6% given semi-solids, which increased to 62.9% and 47.1% by 6 months of age, respectively. At 12 months of age, 98.7% of infants still received breast milk. Non-breast milk liquids given before 6 months of life included tea with milk (8.25%), powdered milk (3.1%), cow's milk (1.5%), and tinned milk (1.0%). At 6 months of age, 0.01% of infants received solid foods compared to 66.9% at 12 months of age. Broad feeding practice categorizations (provision of maternal milk only, maternal milk plus non-breast milk liquids, and maternal milk plus non-breast milk semi-solids)

are depicted according to age in months in **Figure 2.2**. Specific common non-breast milk foods (including liquids and semi-solids) given over the first year of life are depicted by monthly reports in **Figure 2.3.** There were no significant differences in EBF duration based on infant sex or maternal parity.

Figure 2.2. Rural Gambian infant feeding practices by age

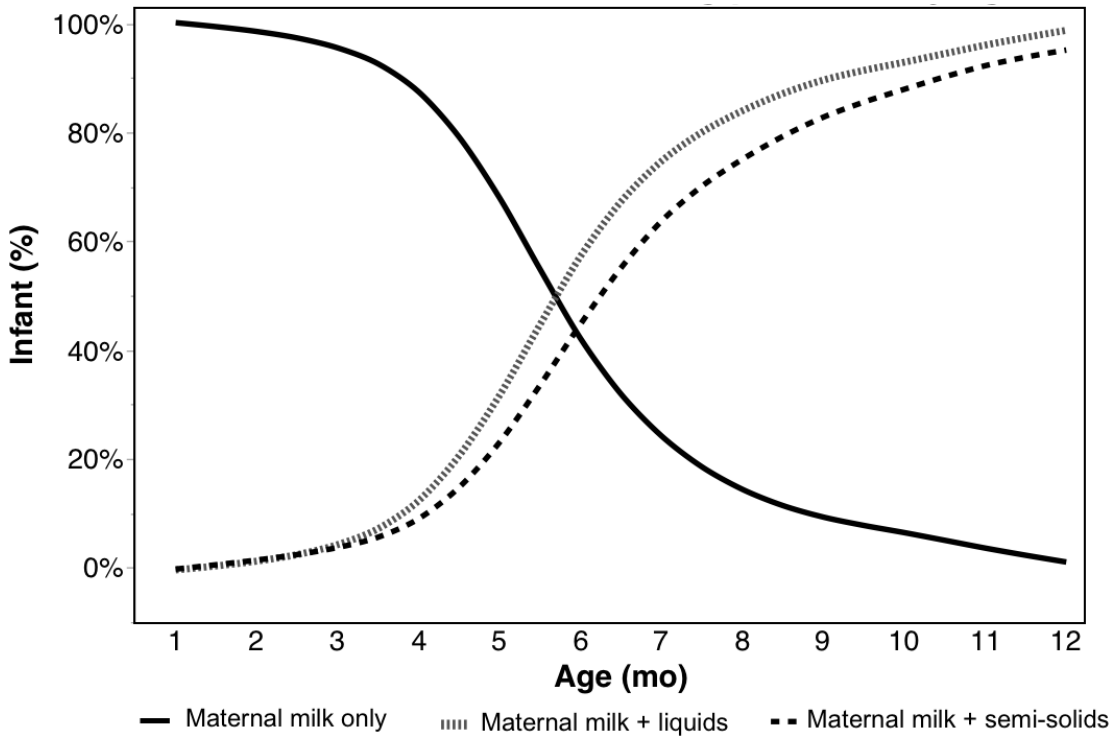


Figure 2.3. Common NBMFs given over the first year of life

Predictors of exclusive breastfeeding duration using multiple linear regression analyses

The multiple linear regression model of predictors of EBF duration had a medium effect size (f^2=0.15). VIF was < 2 for each variable, meeting the criteria for parameters of collinearity. Results of multiple linear regression analyses showed that infant birth month was a significant predictor of exclusive breastfeeding duration in the HERO-G subsample (P=0.0370). Being born in the month of May predicted significantly shorter exclusive breastfeeding duration by -1.68 months (95% CIs: -2.52, -0.84 months; P<.0001) in the multiple linear regression. None of the other birth months were significant predictors of breastfeeding practices. There was no significant predictive effect of infant sex, maternal parity, or household SEP on EBF duration. **Table 2.5** details the statistical model results.

Table 2.5. Multiple linear regression model results of predictors of exclusive breastfeeding duration

EXPLANATORY VARIABLES	B	95% CIs	P
Intercept	5.21	4.71, 5.71	**<.0001***
Birth month			
Jan	0.12	-0.53, 0.78	0.7102
Feb	0.34	-0.31, 1.0	0.3017
Mar	0.24	-0.38,0.87	0.4474
Apr	-0.11	-0.89, 0.67	0.7854
May	-1.68	-2.52, -0.84	**<.0001***
Jun	-0.20	-1.09, 0.70	0.6667
Jul	0.51	-0.44, 1.46	0.2928
Aug	0.71	-0.01, 1.51	0.0846
Sept	0.08	-0.79, 0.96	0.8529
Oct	-0.50	-1.45, 0.46	0.3044
Nov	0.06	-0.84, 0.97	0.8890
Dec (referent)	-	-	-
Infant sex			
Female	-0.14	-0.38, 0.11	0.2699
Parity (continuous)	-0.03	-0.12, 0.07	0.5735
SEP Score (continuous)	0.08	-0.13, 0.29	0.4708

B: Coefficient estimate; 95% CIs: Confidence Intervals (Lower Bound, Upper Bound); P: P-value; *P<.0001; Statistical significance indicated by boldface font/dark gray cell highlight.

Infants born in the month of May (N=13) – shortly before the start of the wet ("hungry") season – had the shortest average exclusive breastfeeding duration at 3.54 (±1.9) months. Infants born in August had the longest average exclusive breastfeeding duration at 5.65 (±1.1) months. Average exclusive breastfeeding durations according to birth month are detailed in **Table 2.6.**

Table 2.6. Average exclusive breastfeeding duration based on birth month

Month	N	Mean	SD	Std Err Mean	95% CIs
Jan	23	5.15	1.09	0.23	4.68, 5.62
Feb	25	5.32	1.06	0.22	4.87, 5.77
Mar	26	5.23	1.38	0.27	4.68, 5.79
Apr	20	4.87	1.68	0.38	4.08, 5.66
May	13	3.54	1.98	0.55	2.34, 4.73
Jun	14	4.96	1.24	0.33	4.25, 5.68
Jul	12	5.39	1.49	0.43	4.45, 6.33
Aug	14	5.65	1.07	0.29	5.04, 6.27
Sept	11	5.09	1.49	0.45	4.09, 6.09
Oct	12	4.47	1.74	0.50	3.36, 5.57
Nov	12	4.97	1.89	0.54	3.77, 6.17
Dec	12	5.43	1.76	0.51	4.31, 6.55

95% CIs: Confidence Intervals (Lower Bound, Upper Bound)

DISCUSSION

While maternal milk can serve as the sole form of nutrition during early infancy, it eventually must be supplemented with non-breast milk foods in order to satisfy the energetic requirements for optimal infant development. In this analysis, 30.4% of rural Gambian infants (N=194) were exclusively breastfed until the WHO recommended 6 months of age. This finding corroborates another recent investigations of breastfeeding practices in a rural Gambian population, where 32% of infants were exclusively breastfed until 6 months of age[264]. Similar findings have been discussed in other studies in populations across sub-Saharan Africa[323,324]. Breastfeeding duration is relatively long in this rural Gambian population, with 98.7% of all infants still receiving maternal milk at 12 months of age, the latest age at which the questionnaire was administered. Other studies have reported breastfeeding to continue until an infant is 18-24 months of age in areas of rural Gambia[223]. The most common non-breast milk foods incorporated into the infant diet during the first year of life in this analysis were grain-based porridges, which include some of the country's staple crops as ingredients.

The multiple linear regression showed a medium effect size. Birth month was the only significant predictor of exclusive breastfeeding duration in the model, with May, the month before the start of the wet season, predicting earlier introduction of non-breast milk foods. The 'maternal work pattern' hypothesis predicts that the introduction of non-breast milk foods and/or breastfeeding cessation will occur earlier in populations where maternal subsistence activities are associated with physical separation of mother and infant for longer durations[36]. During the wet season in The Gambia, mothers commonly spend much of the day separated from their infants, resulting in less frequent breastfeeding and alternative caregivers responsible for infant feeding[36,325]. The results from the present analysis, along with prior studies in the same region, support the maternal work pattern hypothesis in that earlier introduction of non-breast milk foods coincides with the annual period where maternal agricultural workload intensifies. Infants born in May were weaned, on average, by 3.54 (±2.0) months of age, suggesting that those born right before the start of the wet season are likely to cease exclusive breastfeeding before the end of the wet season. These findings align with other studies in this region where maternal workload during the wet season influences infant feeding decisions. However, this finding may also relate to the uneven number of births across different months in the HERO-G cohort. The greatest number of births occurred in January, February, and March in the HERO-G subsample, and the greatest number of infants receiving their first non-breast milk foods in the month of June (which aligns with the median age of weaning

in this study). This may be an artifact of the clustering of recruitment to women for this study at a particular time in the year.

An alternative explanation for the greater number of infants weaned during the month of June is that the number of infant births were not equal across all months of the year; nearly twice as many infants were born in January, February, and March compared to the other months due to the scheduling of recruitment for this study. Thus, this finding may be a byproduct of the greater proportion of births during the early months of the year as opposed to directly stemming from maternal subsistence activities and seasonality. Food availability fluctuates throughout the year in rural Gambia due to seasonal cropping patterns. Food supplies are particularly plentiful in November and December in this region because cereal crops are generally harvested between September and December. Food shortages often occur in July and August[293]. These fluctuations may impact the type and consistency of infant weaning foods offered to infants as complementary foods. By assessing seasonality by month as opposed to the dichotomous wet/dry season categories, we are able to analyze patterns at a finer resolution. Future works should consider investigating 'month' as a continuum rather than category, as using a coefficient of cyclic variation (as in Fourier analysis) could provide an even deeper understanding of the magnitude and temporal patterns at play[326].

Household SEP was not a significant predictor of breastfeeding practices in the multiple linear regression model. This differs from factors established in the literature from other populations, where SEP is associated with earlier EBF cessation due to influences such as limited access to resources such as transportation, access to roads, lower income to purchase nutritionally adequate and hygienic complementary foods, and lower education levels. The present finding challenges that of Issaka et al. (2017), where Gambian children from poor households had significantly higher odds of not meeting the recommendations for timing introduction and type of solids, semi-solids, or other soft complementary foods compared to children from wealthy households. The authors also report that the odds of not meeting the aforementioned dietary requirement increased significantly for children whose fathers were not employed in the agricultural industry. It is important to note that calculation of household SEP was less robust than in other studies due to the reduction of items from the PCA. Acknowledgement of possible bias must be given to the exclusion of livestock ownership in the PCA. Ownership of livestock in The Gambia is predominantly controlled by males in the household, particularly cattle. As such, mothers are unlikely to be the direct owner of these livestock. Because cattle are valuable as sources of food, income, and transportation and traction (e.g., field plowing), ownership of a cow contributes a high score to the SEP calculation. The calculation used to assess household SEP, however, takes

multiple variables into consideration at once, which is more appropriate here than using variables such as maternal education or livestock ownership as single predictor variables.

Other characteristics of this population must be considered in the evaluation of the impact of SEP on exclusive breastfeeding practices in this population. In this region, there is low education attainment and prevalent household crowding. Three quarters of the mothers had no formal education or low formal education (1-7 years) and 173 (85.6%) reported living in crowded households.

Formal education may be less relevant as a driver of infant feeding decisions in this region. Locally designed and run programs such as the Baby Friendly Community Initiative (BFCI) (developed by the National Nutrition Agency) in The Gambia provide local support and education to improve infant and young child feeding practices, including promoting exclusive breastfeeding for 6 months of age[327]. Implementation of the BFCI includes steps such as training, counseling, community meetings, house-to-house visits, provision of sanitary materials and other resources, and continued monitoring and supportive supervision. The villages included in the HERO-G study are among the those implementing the BFCI in the West Kiang. The WHO (2009) reports significant success from implementation of BFCI initiatives in The Gambia with marked improvement in early initiation of breastfeeding and exclusive breastfeeding to 4 months of age. Specifically, the adoption of certain BFCI strategies contributed to the increase in the national average of exclusive breastfeeding from 0% in 1989, to 17.4% in 1998, to 36% in 2000[328]; and 41% in 2006[279].

The Medical Research Council (MRC) has made considerable investments in healthcare and nutrition-related infrastructure in their core study villages in the rural region of the West Kiang; these communities have access to resources such as: ante- and postnatal care, primary health care clinics with trained clinicians and nurses, all children are vaccinated and receive many WHO recommended health interventions (e.g., vitamin A and mebendazole), growth monitoring during early life, treatment for severely malnourished children, and all health services are free. Contaminated open wells and open defecation – present in many settings of poverty in low-income countries around the world – have been replaced with clean piping for water supply and latrines in all compounds. There is also free universal primary education with enrollment at ~97%. Such access to resources and continued individual and community-level health interventions creates a unique situation in rural Africa; government-implementation of such efforts throughout low-income countries is challenging due to required expenses.

There were some limitations to this study. First, collection of retrospective dietary intake data may introduce bias through recall error[329–331]. However, studies comparing maternal recall methods found that 24- and 48-hour recall periods resulted in overestimated exclusive breastfeeding durations, and report that 7 to 10-day recall intervals are adequate for general assessment of food intake, and may more accurately capture the complexity of infant feeding patterns compared to shorter intervals[331–333]. Future analyses may benefit from analyses of dietary diversity scores, hygienic index calculations, details of ingredients and preparation processes for homemade weaning foods, assessment of food volumes, focus groups on local feeding decisions/motivations, in particular the transfer of knowledge between generations/sources of information that inform maternal decision to introduce non-breast milk foods. Future study designs may consider adopting methods to assess any specific relationships between infant weaning practices and shifts in availability of certain food ingredients and/or maternal workload across the seasons.

A diverse diet incorporating a variety of food types has been recommended as an approach to achieving adequate nutrient intake. Many household dietary surveys such as these also examine features of dietary diversity, which is commonly used as a marker of nutritional wellbeing in the guidelines of many country nutritional assessments. A dietary diversity score (DDS) may be another useful approach to investigating complementary feeding practices in future evaluations. DDS is calculated by summing the number of unique food groups (cereals and tubers, dairy, eggs, vegetables, fruits, oils and fats, meat, etc.) consumed by an individual within a specified period of time. The types of complementary foods used in this subsample were indicated by the mothers. As reported in the results, it was most common to use cereal-based gruels as complementary foods. However, because the questionnaires were collected over the first year of life, a period when solids are used less frequently compared to semi-solids (66.9% of infants received solids whereas 94.7% received semi-solids at 12 months of age), implementing a DDS here may not be most the most appropriate method. Future analyses may consider incorporating DDS as a methodology to assess potential shifts in early life diet based on seasonal effects on food supply. This may also be effective in large scale comparisons using national-level data sets.

Infant health and growth attainment may impact a mother's decision to wean her infant earlier. Research on the directionality of these associations, however, provides mixed results. For example, mothers may cease exclusive breastfeeding or modify feeding practices because their infants are ill, perhaps related to hospitalization, type of illness, or perception that maternal milk is not meeting the infant's immunological needs[158]. Others may wean infants perceived as healthy (e.g., fewer morbidities) earlier, which may result in increased incidence of morbidity as it relates

to reduced immunological protection from maternal milk. Infants perceived as growing well may receive non-breast milk foods earlier if they appear to demand more feeding; mothers may also exclusively breastfed for longer durations if they regard breastfeeding as causal to healthy growth[264]. These alternative perceptions may bias the order of causality and lead to overestimation of the immediate protective effects of exclusive breastfeeding. Thus, infant feeding practices directly prior to the onset of an illness or growth faltering event may be a better indicator of the effects of breastfeeding in relationship to infant morbidity and growth. These relationships are explored in Chapters 4 and 5.

CONCLUSION

The goal of Chapter 2 was to characterize infant breastfeeding and complementary feeding practices during early life in a subsample of mother-infant pairs (N=194) from the larger HERO-G cohort. Through use of maternal reports of exclusive breastfeeding duration and infant complementary food type and use, I found that cessation of exclusive breastfeeding occurs around 5 months of age in this subsample. Maternal milk remains an important part of the infant diet, with nearly all infants continuing to receive some maternal milk at the 12 month time point. Assessment of potential predictors of exclusive breastfeeding duration showed that seasonality may be an important driver of the timing of introduction of non-breast milk foods into the infant diet. This may relate to maternal agricultural workloads, which fluctuate with the annual rains and may influence infant caregiving and subsequently infant feeding practices.

Ultimately, the dietary intake during early life and the feeding practices caregivers implement while offspring are too young to feed themselves are important in contextualizing subsequent health and growth outcomes. Diet over the weaning period represents a useful framework in understanding the impact of first foods on short and longer term health and growth phenotypes, and features of lactation, such as exclusive breastfeeding duration, may serve as proximate indicators of available maternal resources for investment in offspring. Here, the characterization of infant breastfeeding and complementary feeding practices during early life in a population under considerable environmental and physiological stress sets the foundation for the analyses in the following Chapters, in which I apply the dietary contexts reported here to analyses of human biological variation in maternal lactation strategies and infant health and growth outcomes.

CHAPTER 3. MATERNAL MILK COMPOSITION

INTRODUCTION

Human milk composition is complex, dynamic, and highly variable; it has evolved over millions of years to meet the nutritional and immunological needs of the developing infant. There is considerable variation in both milk production and composition across populations, geographic regions, and between individual mothers. Milk composition varies according to the time of day, stage of lactation, and within and between feeds.

In this Chapter, I report on measurements of maternal milk composition, including macronutrient (fat, protein, lactose, and true protein) concentrations, and the relative abundances of human milk oligosaccharide (HMO) classes (fucosylated, sialylated, undecorated, and sialylated and fucosylated), individual HMO structures, and two human milk glycoproteins (HMGPs), lactoferrin and immunoglobulin A (IgA), across the first 12 months of lactation, in a subset of individuals (N=50) from the HERO-G subsample. I then contextualize discussion of these results, and of variation in milk composition, within a life history framework.

BACKGROUND

Human milk composition

Human milk macronutrients

Milk macronutrients, including fat, protein, and carbohydrates, vary within and between mothers and across lactation. However, evidence suggests that macronutrients are generally conserved across populations despite variations in maternal nutritional status and environmental stressors[177,334]. There are some general trends in compositional shifts of milk macronutrients across the course of lactation in humans. These include a gradual decline in protein and lipids over the first 6 months of lactation and an increase in lactose from colostrum and transitional milk to mature milk[138,153]. The ratio of whey to casein proteins changes from approximately 80:20 in early lactation to around 50:50 in late lactation[154].

Fat concentration (average in mature, term milk: 3.2-3.6g/dL) is the most variable macronutrient in milk, and fluctuates across the lactation period[335–338]. Maternal diet is a determinant of fatty acid profiles in milk, likely due, in part, to the nature of fats consumed by mothers[114,336,339,340]. In fact, maternal milk fat composition mimics that of dietary fat within 2 days of consumption by mother[156]. Fat content in milk collected from malnourished mothers has

been shown to be reduced; however, dietary supplementation in mothers in The Gambia did not result in significant changes to the milk composition[341].

Mature milk contains around 0.9-1.2 g/dL of protein. Milk proteins can be grouped into the whey or casein protein fractions, which are each composed by a multitude of specific proteins and peptides[157,158]. True protein quantifies only the proteins in milk (whereas total protein, here referred to as 'protein') is a measure of all sources of nitrogen and includes non-protein nitrogen, such as urea, which has no nutritional value to humans[342]. True protein is determined by taking the entire nitrogen content in milk minus the non-protein nitrogen fraction. Protein concentrations do not appear to be impacted by maternal diet, but increase with maternal BMI[159].

The principal carbohydrate in maternal milk is lactose, which is the least variable of the macronutrients. Around 40% of the calories in human milk are provided by lactose. It also promotes healthy gut microbiota composition, insulin regulation, and the production of antimicrobial factors in the gut.

Human milk oligosaccharides

Human milk oligosaccharides (HMOs) are the third most abundant solid in milk after lactose and lipids. HMOs are complex sugars found in human milk (more than 200 HMOs with unique structures have been identified in human milk[343]). They are a structurally and biologically diverse group of sugars which are indigestible to the infant. Research on HMO metabolism in infants has shown that around 1% of HMO content is absorbed into infant circulation and the rest are consumed by gut microbes or excreted in feces or urine[344]. Still, HMOs have been shown to directly contribute to offspring health and development in numerous ways. For example, they can protect against infection and disease[345–347] and support neurological development[348].

HMO synthesis follows a basic blueprint, with all HMOs containing five basic monosaccharides: glucose (Glc), galactose (Gal), N-ethylglucosamine (GlcNAc), fucose (fuc), and sialic acid (SA). All HMOs are based on a lactose molecule (disaccharide compose of Gal bonded to Glc molecule). As such, HMO biosynthesis is likely an extension of lactose synthesis. The four main HMO classes (grouped based on monosaccharide composition) that will be analyzed in this dissertation are: fucosylated (structure with fuc), sialylated (structures with SA), sialylated and fucosylated (structures with both SA and fuc), and undecorated (those lacking both fuc and SA).

Human milk glycoproteins

Human milk glycoproteins (HMGPs) contain sugar chains structurally similar to HMOs[186–188]. HMGPs are less digestible and of less nutritional value than other protein types[19,192,197–199]. Instead, they play a key role in immune defense. Of the more than 400 HMGPs that have been characterized in human milk, those whose activities are most widely recognized in research to date include both innate immune proteins (lactoferrin, lysozyme, and a-lactalbumin) and acquired immune proteins (s/IgA, IgG, IgM)[343,349]. The concentration of HMGPs increases six-fold during mammary involution during weaning from prolonged nursing, which provides the breast with protection against infection and inflammation[151]. This increase in proteins is also beneficial to toddlers who may become ill during the weaning period[23,152]. In this dissertation, I focus on the relative abundance of milk lactoferrin and immunoglobulin A (IgA).

Lactoferrin is the second most abundant protein overall and most dominant whey protein in human milk and is present in high quantities[350]. It plays a critical role in infant innate immune system; it can regulate the maturation and function of immune cells and recruit them to sites of infection[350]. In particular, lactoferrin has been observed to protect against certain gastrointestinal infections in breastfed infants[351,352]. Because of its iron-binding properties, it can reduce the availability of iron to pathogenic bacteria in the gastrointestinal tract[353–355]. Evolutionarily, the naturally occurring low iron concentrations in maternal milk combined with depleted infant iron stores at the time of weaning may have improved offspring survival by reducing proliferation of pathogenic gut bacteria which infants are more highly exposed to during the transition from exclusive breastfeeding to novel complementary foods[149]. Thus, lactoferrin is particularly important to the health of the infant, since it appears to utilize iron stores, thus restricting iron available for growth of pathogens such as *E. coli* and *Staphylococcus.* For example, the secretion system of enteropathogenic *E. coli*, a leading cause of diarrhea in low-income countries, is blocked by lactoferrin via degradation of particular proteins used by the pathogen to adhere to host cells (particularly epithelial cells in the GI tract)[356]. In low-income populations, where diarrheal disease and other morbidities are common and significantly increase risk of mortality in children under 5 years of age, the defenses provided by lactoferrin in maternal milk are important[87,357–361].

Immunoglobulin A (IgA) is an antibody involved in immune defense. Similar to lactoferrin, it has functionalities in pathogen binding inhibition. IgA also has anti-inflammatory effects[362–369]. IgA is implicated in the efficiency of mucosal barriers throughout the body, including the GI tract. IgA is abundant in human milk, ranging

from around 6–15% of total milk protein with considerable between-population variation[370–372]. IgA is an example of a direct maternal investment in infant health, helping coat and protect the infant GI tract from infection while the infant develops the ability to produce IgA on their own. Milk IgA may be sensitive to maternal energy fluctuations.

Maternal factors influencing milk composition

Maternal diet

In the first few months of life, the nutritional demand for lactating mothers is increased to support offspring growth and to maintain her own metabolism. Lactating women who do not have an adequate dietary intake risk depleting their energetic and micronutrient stores, which can negatively affect the nutritional status of the breastfeeding infant or child. Undernutrition in women, before and during pregnancy, is recognized as a key determinant of poor pregnancy outcomes including poor fetal development, preterm births, and small for gestational age and low birth weight infants, often leading to increased infant morbidity and mortality[373]. Limited resource availability, due to factors such as food scarcity and/or increased household demand or financial burdens, would be expected to negatively impact either maternal milk production or composition[374]. However, unless extremely severe, maternal undernutrition does not necessarily pose a risk for breastfed infants in environments where maternal dietary intake is low. Mothers experiencing exceptionally severe chronic nutritional or physical stress can produce milk lower in energy content, and, in cases of extreme undernutrition, milk volume may be significantly reduced or complete cessation of milk production may occur[375]. Older studies have found that undernourished women only produced half the volume of milk produced by well-nourished women from more affluent countries; however, subsequent work using improved methods showed no distinction in the production of milk volume between undernourished and well-nourished mothers[341,376,377].

In the existing literature, relationships between nutritional status/diet and maternal milk composition are commonly examined in "real time," where present dietary intake and/or maternal anthropometry are compared to present milk composition. Most evidence suggests that milk macronutrient composition of human milk is conserved across populations and is independent of nutritional status. For example, previous studies investigating the influence of maternal diet and nutritional status on milk composition during lactation have found that milk protein concentrations are generally not impacted by maternal diet[106,113]. However, one intervention study in rural Gambia reported a small increase in maternal milk protein and a decrease in lactose concentration with a high energy dietary supplement given

during lactation[115]. In some studies, lactose, the main carbohydrate found in maternal milk, was not influenced by supplements[378–380]; however, one study found a modest, inverse association between maternal adiposity and milk carbohydrate concentrations[381]. Seferovic et al. (2020) report that relative to a high carbohydrate diet, a diet high in fat decreased the concentrations of sialylated HMOs[382]. A study of lactating women who fasted during Ramadan reports no significant effect on macronutrient composition of maternal milk but did find significantly lower concentrations of zinc, magnesium, and potassium[383]. Some evidence shows that abundances of HMOs and HMGPs are influenced by dietary intake. For example, a study reports that vitamin A intake was associated with increased sialylated HMOs[384]. Distinct maternal dietary carbohydrate and energy sources during lactation appear to preferentially alter fucosylated HMO abundance in milk[385]. During lactation, rural Gambian women produce less milk IgA during the wet ("hungry") season compared to the dry ("harvest") season, suggesting that IgA production is sensitive to seasonal changes in food availability, a factor capable of impacting nutritional status[386].

Evidence to suggest that diet near the time of conception and during pregnancy can impact human milk composition is limited and mixed[387–389]. Some studies show that vitamin A intake and serum vitamin A concentrations during pregnancy influence the composition of maternal milk[388]. Similarly, dietary supplementation of long-chain polyunsaturated fatty acids from week 30 of gestation and onward more than tripled the fatty acid content in milk produced during early lactation; supplementation limited to pregnancy only was much less effective[387]. In a population from Finland, supplementation of maternal diet with probiotics from 36 weeks of gestation until birth was linked with increased 3'FL and 3'SL levels in colostrum[390]. Low vitamin B_{12} intake during pregnancy (first, second, and third trimesters) and lactation was associated with low concentrations of B_{12} in rural Kenyan maternal milk[391]. Alternatively, calcium supplementation of pregnant Gambian women had no significant impact on breast-milk calcium concentrations in the first year of life[392]. In a population from Italy, consumption of eggs and fish during late stages of pregnancy was weakly correlated with selenium in maternal milk during the first month of lactation, but very few of exclusively breastfed infants did not meet the recommended daily selenium intake[393].

Studies assessing relationships between maternal nutritional status during pregnancy and milk composition across the first year of lactation are lacking. However, the available data suggest some connections exist. For example, studies have found a positive association between maternal pre-pregnancy BMI and concentrations of fucosylated HMOs (particularly 2'FL) and total HMO abundance in human milk[394–396]. Additionally, maternal MUAC during the third trimester was positively associated with milk lactoferrin concentration during the first month of lactation in one

Indonesian population[397]. Maternal nutritional status during pregnancy may also influence milk volume. Specifically, a study in pastoral communities in Kenya reports a significant positive relationship between maternal MUAC during the third trimester and infant milk intake at 4 months of age[398].

Parity

A mother's physiology and reproductive history can influence her milk composition. Primiparous mothers (mothers with no previous births), for example, are more likely to experience difficulties with lactation during the first few days post-partum[399]. Primiparous mothers have also been shown to produce milk with higher concentrations of macronutrients than multiparous mothers (mothers with previous births)[400,401]. Associations between parity and HMO composition in maternal milk vary[402]. For example, there are some reports of higher abundance of LNnT and lower abundance of 3'FL in the milk of multiparous mothers[394,403], whereas others have observed the opposite pattern[404]. Parity has been both positively and negatively associated with milk lactoferrin concentration[405,406]. Others have found no relationship between the two[188,407].

Variation observed in maternal milk composition between mothers of different parity may relate to differences in mammary gland development, which is regulated during puberty and pregnancy by reproductive hormones such as estrogen, prolactin, progesterone, placental lactogen) and metabolic hormones (including growth hormone, insulin, and leptin)[408,409]. These reproductive and metabolic hormones also regulate milk output. Mammary gland development during pregnancy is nearly identical in both primiparous and multiparous mothers; however, transcriptional levels of gene expression in alveolar epithelial cells before conception and ductal morphogenesis (the change in structure and function of ductal glands during the gestation period), are significantly different[408,410,411]. The impact of metabolic hormones on mammary gland development is likely influenced by early environmental and physiological cues, such as body composition and diet, may impact milk composition and output in adulthood[408,410,412,413].

Maternal genetics

Milk composition, particularly HMO abundance, is influenced by maternal genetics. Secretor status (being a 'Secretor' or 'Non-Secretor') refers to the presence or absence of the water-soluble form of the ABO blood group antigens in bodily fluids, such as saliva, tears, and breast milk. The presence and abundance of different HMOs in

human milk are genetically determined and are closely related to Secretor state because HMO production depends on blood group antigens[403]. Thus, maternal Secretor status is used in most studies as a confounding variable when assessing drivers of HMO composition[394,404,414]. Maternal Secretor status can be determined by genotyping the presence of fucosyltransferase 2 gene (*FUT2*) Secretor genes using polymerase chain reaction-random fragment length polymorphisms[415]; however, many studies use milk phenotype (in this case, HMO structure profiles) as a proxy for Secretor status. In most populations that have been investigated, the majority of mothers express *FUT2*, which links Fuc to terminal Gal in an α1-2 linkage[416]. The designation of 'Secretor' versus 'non-Secretor' is determined by the proportion of α1-2-linked fucosylated HMOs: mothers with the Secretor phenotype are *FUT2* positive and produce milk containing a higher proportion of α1-2-linked fucosylated HMOs compared with women with the non-Secretor phenotype[189,416,417]. In studies where genotype and phenotype are examined, these HMOs are virtually absent in the milk of non-Secretor mothers who do not express *FUT2*[418]. As such, the abundance of 2'FL in maternal milk is an accepted indicator of Secretor status. A relative cutoff of >6% α1-2 fucosylation is commonly used in milk HMO research[419].

Many publications cite that around 80% of mothers have the active *FUT2* gene. However, growing evidence shows global variation in the proportion of mothers who Secretors versus non-Secretors. Reported proportions of Secretor mothers include 67–95% in the United States[196,420,421], 64–87% in the United Kingdom, Spain, Finland, Sweden and Italy, 85% in India, 77% and 80% in regions of China[422], 89.1% in a Brazilian cohort[423], and 51–81% in Africa (Burkina Faso, Ethiopia, Ghana, Kenya, South Africa, and Malawi)[189,424]. In the existing studies from The Gambia, Secretor prevalence was 85% in an urban population and rural populations have reported prevalence of 65% and 73%[424,425]. The adaptive significance of this variation is still being explored. Below, I present a table summarizing some of the costs and benefits of mothers having non-Secretor *FUT2* genotype.

Table 3.1. Examples of evolutionary tradeoffs of having non-Secretor *FUT2* genotype for mother

Benefits	**Costs**
Norovirus resistant	Higher risk of *E. coli* urinary tract infection
HIV resistant	Higher risk of flu virus infection
Reduced risk of H. pylori infection	Higher risk of rheumatic fever
Reduced angiogenesis	Higher risk of cholera
	Greater susceptibility to Chron's disease
	Higher risk of Type 1 diabetes

Infant sex

Male and female infants may have different nutritional requirements for optimal growth and development, which may be reflected in maternal milk composition. In some studies, human milk energy content varies between mothers of male and female infants, but no associations were found between infant sex and milk macronutrient composition in other investigations[394,426–428]. Two investigations have observed higher Lacto-N-hexaose (LNH) concentrations in milk produced by mothers of male infants[394,428]. Further studies are required to assess possible sex-based differences in milk composition profiles and to determine underlying mechanisms.

Lactation stage

Maternal milk composition varies across the course of lactation. Colostrum, the first milk produced, tends to contain lower lactose concentrations[429] and greater concentrations of immunological molecules[368] compared to mature milk. Protein content in milk gradually declines throughout lactation, whereas fat increases[430,431].

Mothers synthesize different subsets of HMO structures, and the total amount and relative abundance of HMOs change over the course of lactation[418]. Total HMO concentrations are highest at the start of lactation and generally continue to decrease over time[404,423,432,433]. In particular, there is some evidence of higher enzymatic activity of *FUT2* in early lactation[404,434]. However, some studies have observed greater abundances of certain HMO structures in milk collected later in lactation[196,420,435].

HMGPs such as IgA and lactoferrin tend to decrease rapidly during early lactation. This is perhaps a reflection of specific mammary gland mechanisms that produce HMGP content according to maternal conditions and/or infant needs[436]. This pattern is not seen in all populations, however. In one study, IgA concentrations from a Gambian population remained relatively stable as opposed to decreasing during the early months of lactation[386]. This is perhaps due to chronic inflammation or selective environmental pressures on the maternal immune system in this particular region.

Previous research on human milk composition in The Gambia

Much research has been conducted on maternal milk composition in The Gambia. Research investigating maternal milk macronutrients show reduced levels of fat, protein, and slightly higher levels of carbohydrates in milk

samples from Gambian women collected during the wet ('hungry') season relative to the dry (harvest) season[171,172]. This may reflect a maternal strategy to reduce overall energy expenditure in milk production in order to more effectively sustain maintenance and repair processes within her own body[171]. A 1984 study measured milk immune proteins (including immunoglobulins A, G, and M, and lactoferrin and lysozyme) from milk collected from rural Gambian mothers up to 26 months lactation found that concentrations of these immune proteins decreased during the first year of lactation, except lysozyme, which increased progressively over time[173,174]. The authors also report that IgG and IgM were higher, and lysozyme lower, in the milk of Gambian mothers than in a population from the UK, but IgA and lactoferrin concentrations were similar in composition between the populations.

Using the same dataset, Prentice et al. (1984b) found no increase in immune protein concentrations during times of high infectious disease load in children, including during cases of diarrhea. However, there was evidence of a slight increase in certain immune proteins during times that indicate a temporally-linked relationship between skin sepsis in infants and maternal milk immune protein composition. Additionally, mothers with lower parity produced higher concentrations of immune proteins compared to those with three or more children. Research in 2004 found that maternal milk IgA levels were higher in Gambian mothers of infants with no evidence of *Helicobacter pylori* (*H. pylori*) colonization, suggesting that antibodies in mother's milk can protect infants against colonization of certain pathogenic bacteria in early life[175].

Investigations of milk HMO in rural Gambian populations are in early stages but show connections between infant health and growth outcomes. Davis et al. (2017) found that mothers in this environment produced more HMOs in the dry season compared to the wet season. The authors speculate that higher energy intake during the dry season may be responsible for higher HMO synthesis. Evidence suggests beneficial effects of fucosylated HMOs on infant health in The Gambia, whereby infants consuming milk with greater abundances of fucosylated HMOs experienced fewer morbidities at four months of age[437]. HMO structures such as LNDF I may have specific protective effects against Group B *Streptococcus* infection in mothers and their infants[438].

RESEARCH AIMS

Aims

The aim of this Chapter is to investigate maternal, infant, and environmental factors that may influence maternal milk macronutrient, HMO, and HMGP composition. I report on measurements of milk composition in a

subset of the HERO-G subsample, including macronutrient (fat, protein, lactose, and true protein) concentrations, relative abundances of HMO classes (fucosylated, sialylated, undecorated, and sialylated and fucosylated [sia-fuc]) and HMGPs (lactoferrin and IgA). Next, I assess potential predictors of milk composition at 3, 6, 9, and 12 months of lactation. I contextualize these results within a broader evaluation of some potential drivers of variation in milk composition.

METHODS

Sample subset

A subset (N=50) of the HERO-G subsample was selected for the milk composition analysis (see Chapter 2 for selection criteria for the N=194 HERO-G subsample). The subset of the HERO-G subsample will herein be referred to as the 'milk analysis subset'. Sample size was bound by laboratory processing constraints. Individuals were selected based on milk sample availability (i.e., sample present from each collection time point and adequate milk volume available for laboratory analyses) and completeness of other contextualizing data (e.g., infant anthropometric measurements and data from clinic health visits present, which will be investigated in Chapters 3-5). Post hoc statistical power of a sample size of 50 (per time point) in examining associations between maternal milk composition and maternal, infant, and environmental factors using multiple linear regression (F-test), was conducted. With a medium effect size (f^2=0.15) and a significance level of $\alpha = 0.05$, a multiple linear regression with 7 predictors and a sample size of 50 (per time point) has a power (1-ß err prob) of 0.89. The power analysis was conducted using G*Power 3.1[316].

Socioeconomic position

Fieldworkers administered a socioeconomic questionnaire during the booking visit for the HERO-G study. Mothers were asked to provide information regarding sociodemographic variables (maternal education attainment), household characteristics (crowding index [number of persons per room within a dwelling], material of dwelling walls and floor), and durable assets (livestock ownership, possession of a cart). Questionnaire responses describing sociodemographic characteristics, household characteristics, and durable assets were used to generate an asset score using Principal Component Analysis (PCA). Complete details on the socioeconomic position (SEP) calculation are

provided in Chapter 2. The same inclusion/exclusion criteria were used and scores were calculated specifically for the HERO-G milk analysis subset.

Maternal mid-upper arm circumference (MUAC) measurements

Maternal anthropometric measurements, including MUAC (see Chapter 1 for full details on the HERO-G study anthropometric data collection), were collected at 20, 28, and 36 weeks of gestation (*Note:* HERO-G study participants who were < 20 weeks pregnant were seen at a 'booking' visit [see Chapter 1], and then at 20, 28 and 36 weeks' gestation. Participants who were > 20 weeks pregnant were seen at booking, 28 and 36 weeks only). At the scheduled prenatal clinic visit, a HERO-G study midwife measured maternal MUAC using flexible measuring tape (Seca 212) to the nearest 0.1 mm[263]. Undernutrition was classified in this dissertation as a MUAC value of < 23 cm[265]. Here, maternal MUAC measurements collected at 36 weeks (third trimester) were incorporated into the analyses as a general proxy of maternal condition/nutritional status near the start of lactation. Existing literature demonstrates that MUAC measurements collected in the second and third trimester are sufficient markers of maternal undernutrition[266,267].

Maternal milk collection

During scheduled clinic visits (3, 6, 9, and 12 months post-partum), mothers were asked to nurse their infant for 2-3 minutes and then hand-express 10mL of mid-feed milk (5mL from each breast) into sterile plastic tubes. Where possible, samples were collected at approximately 1:00pm. All samples were subsequently divided into smaller aliquots and kept frozen at -80°C. Time since last feed and time of sample collection were also recorded. The total volume of milk consumed by infants each day was not measured as a part of the HERO-G project. All samples selected for this analysis were expressed from the right breast order to maintain consistency in analysis. Additional details of the HERO-G maternal milk collection protocol can be found in **Appendix Table A.1.**

Human milk macronutrient analysis

Fat, lactose, total protein (protein), and true protein (TRP) concentrations were measured in maternal milk samples (N=200) collected at 3 (N=50), 6 (N=50), 9 (N=50), and 12 (N=50) months post-partum using the LactoScope FTIR (Perten Instruments, Inc.), which employs Fourier Transform Mid-Infrared Spectroscopy, in the Growth and

Development Laboratory at the University of Colorado Boulder. Samples were prepared for analysis by diluting 2mL of each milk sample 10x by adding 18mL of ddH_2O. The diluted samples were warmed to 38.5°C in a water bath prior to analysis for no longer than 15 minutes. Each sample was run in duplicate.

Human milk glycoprotein analysis

Triple-quadrupole time-of-flight (TOF) mass spectrometry (MS) with an Agilent 6520 Q-TOF MS (Agilent Technologies, Inc.) was conducted at the Lebrilla Laboratory at the University of California Davis to measure relative HMGP (lactoferrin and immunoglobulin A) abundance in maternal milk samples, with all preparation and analyses following a validated protocol, and HMO abundance using a nano-high performance liquid chromatography (HPLC)-chip/TOF mass spectrometer[439]. MS quantitates analytes by measuring mass-to-charge ratio (m/z) of ionized molecules, and can provide greater analytical sensitivity and specificity than other methods such as immunoassay, especially at low concentrations. MS output plots show the different m/z against their abundances (occurrence of a certain ion divided by the occurrence of the most abundant ion) within each sample.

Human milk oligosaccharide analysis

Free HMOs were extracted from whole milk samples following previously reported/validated methods[439] at the Lebrilla Laboratory at the University of California Davis. 50μL of each milk sample was aliquoted onto 96-well plates, diluted, and defatted via centrifugation. The resulting glycans were reduced with 1.0 M NaBH4 in a water bath at 65 °C for 1.5 hours. The samples were then purified on solid phase extraction graphitized carbon cartridges (GCC). HMO samples were loaded onto the GCCs, desalted with deionized water, and eluted with 20% acetonitrile in water and then 40% acetonitrile in 0.05% trifluoroacetic acid (v/v). The eluent fractions were combined and the solvent evaporated.

Analysis of the extracted HMOs was performed on a nano-high performance liquid chromatography (HPLC)-chip/TOF mass spectrometer. The HMO samples were loaded onto the enrichment column (1 μL injection) by the capillary pump at a flow rate of 4.0 μL/min. Separation was achieved with a binary gradient of aqueous solvent A (3% acetonitrile/water (v/v) in 0.1% formic acid) and organic solvent B (90% acetonitrile/water (v/v) in 0.1% formic acid). Data was collected in the positive mode, and the instrument was calibrated by a dual nebulizer electrospray source with internal calibrant ions ranging from *m/z* 50-3000.

Data were collected and analyzed using Agilent MassHunter Qualitative Analysis software. Specific HMO structures were identified and assigned by matching retention time and exact mass, within 20 ppm mass-error, to calculated masses in previously annotated HMO libraries. Absolute abundances in ion counts were directly correlated to abundance of the compounds present. Glycan types were divided into four classes based on monosaccharide composition: fucosylated (structure with fucose), sialylated (structures with Neu5Ac), sialylated and fucosylated (structures with both sialic acid and fucose), and undecorated (those lacking both fucose and sialic acid). Relative abundance of each class was determined by dividing abundances by the total oligosaccharide count for each mother. The same calculation was used to determine relative abundance of individually identified compounds. Relative concentrations are given as percentage of total HMOs.

Secretor status

Secretor status ('Secretor' vs 'non-Secretor') was designated based on relative abundance of known α1–2-linked fucosylated HMOs, including 2′FL, LDFT, TFLNH, DFLNHa, DFLNHc, and IFLNH I (see Table 3.21 for full list of HMO structure names) in each sample. Mothers who produced milk containing >6% relative abundance of α1–2-linked fucosylated HMOs were categorized as phenotypic Secretors[419].

Infant feeding practices

Dietary questionnaires regarding infant feeding were administered to mothers by trained field workers every 10 days starting at one week of infant age until 12 months of infant age. Mothers or caregivers were asked to recall infant feeding practices in the previous 10 days. Infant feeding practice was defined by exclusive breastfeeding status at 6 months of age, based on the WHO recommended exclusive breastfeeding duration of 6 months of age[321]. Infants were categorized as either 'EBF <6mo' (provision of breast milk and non-breast milk foods/liquids before 6 months of age) or 'EBF ≥6mo' (provision of breast milk only until 6 months of age or later)[264,322]. Additional details regarding infant dietary questionnaire methodology can be found in Chapter 2.

Statistical analysis

Sample size

Maternal milk macronutrient composition was measured in a total of 200 samples selected from time points across the first 12 months post-partum, with 50 samples from collections at 3, 6, 9, and 12 months of lactation.

Statistical methods

Concentrations and relative abundances of the milk constituents measured in this analysis are reported with mean, standard deviations (SD), and ranges where appropriate. Mixed effects models were constructed to assess potential predictors of milk composition during first year of lactation, taking into consideration the possible influences of EBF duration, demographic (household SEP), maternal (parity, Secretor status), infant (sex) and environmental factors (birth season, season of milk collection). One mixed effects model was constructed for each individual milk constituent. Multiple linear models were also constructed to assess potential "real time" and "extended" predictors of each individual milk constituent at 3, 6, 9, and 12 months post-partum. Here, "real time" predictors are assessed using dietary variables (EBF status) at a collection time point in relationship to milk composition at that same time point (e.g., EBF status at 3 months of age in model assessing milk composition at 3 months of lactation). "Extended" predictors are assessed using dietary conditions (milk composition and EBF status) at earlier time points in relationship to milk composition at later collection time points (e.g., milk composition at 3 months of lactation and EBF status at 3 months of age in model assessing milk composition at 12 months of lactation). **Table 3.2** details the explanatory and response variables incorporated into the models. Descriptive statistics were calculated for all variables included in the statistical models. Multicollinearity was determined using a conservative VIF > 5 in multiple linear regressions. Four multiple linear regression models were run for each milk constituent: one for composition at 3, 6, 9, and 12 months separately. Significant associations identified in both the mixed effects models and multiple linear regression models were summarized using the beta regression coefficients (B), 95% confidence intervals (CI), and P-values. Where appropriate, model effect size is reported as Cohen's f^2, where $f^2 \geq 0.02$, $f^2 \geq 0.15$, and $f^2 \geq 0.35$ represent small, medium, and large effect sizes, respectively[440]. Associations between all maternal milk macronutrient concentrations, and relative abundances of HMO classes and HMGPs were assessed using correlation matrices. Correlations between milk constituents at 3, 6, 9, and 12 months of lactation are reported as R^2 values. An R^2 value >0.80 was considered a significant correlation. Wilcoxon Signed-Ranks Test was used in post-hoc investigations of

seasonal differences in maternal milk composition. The level of statistical significance was set to $P < 0.05$ for all analyses. All statistical analyses were conducted using JMP Pro 15.0 statistical software (©2019 SAS Institute, Inc.).

Table 3.2. Response and explanatory variables investigated in separate mixed effects models for each milk constituent

	Outcome variable	Explanatory variable
Macronutrients	Fat	*Fixed* ● Maternal MUAC[a] (continuous) ● Infant sex (F/M) ● Season of milk collection (Wet/Dry) ● Infant season of birth (Wet/Dry) ● Socioeconomic position (SEP score) ● Parity (continuous) ● EBF duration (months) ● Collection time point (3, 6, 9, 12mo) *Random* ● Subject ID
	Protein	
	Lactose	
	TRP	
HMOs & HMGPs	Fuc	*Fixed* ● Maternal Secretor status ● Maternal MUAC[a] (continuous) ● Infant sex (F/M) ● Season of milk collection (Wet/Dry) ● Infant season of birth (Wet/Dry) ● Socioeconomic position (SEP score) ● Parity (continuous) ● EBF duration (months) ● Collection time point (3, 6, 9, 12mo) *Random* ● Subject ID
	Sia	
	Undec	
	Sia-fuc	
	LF	
	IgA	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy; Fuc: Fucosylated HMO; Sia: Sialylated HMO; Undec: Undecorated HMO; LF: Lactoferrin

Table 3.3. Response and explanatory variables investigated in separate multiple linear regressions at 3, 6, 9, and 12 months post-partum for each milk constituent

	Outcome variable*	Explanatory variable
Macronutrients	Fat	● EBF status at 3mo or 6mo (Y/N) ● Maternal MUAC[a] (continuous) ● Infant sex (F/M) ● Season of milk collection (wet/dry) ● Infant season of birth (wet/dry) ● Socioeconomic position (SEP score) ● Parity (continuous)
	Protein	
	Lactose	
	TRP	
HMOs & HMGPs	Fuc	● Maternal Secretor status ● EBF status at 3mo or 6mo (Y/N) ● Maternal MUAC[a] (continuous) ● Infant sex (F/M) ● Season of milk collection (wet/dry) ● Infant season of birth (wet/dry) ● Socioeconomic position (SEP score) ● Parity (continuous)
	Sia	
	Undec	
	Sia-fuc	
	LF	
	IgA	

*Outcome variables were assessed separately at the 3, 6, 9, and 12 month time points; [a]Maternal MUAC measurements were collected during the third trimester of pregnancy; Fuc: Fucosylated HMO; Sia: Sialylated HMO; Undec: Undecorated HMO; LF: Lactoferrin

RESULTS

Maternal and infant characteristics

Average (±SD; range) maternal age in this subset of study participants (N=50) (milk analysis subset) was 31.9 (±6.5; 19.5-42.3) years. Because 48 (96.0%) of mothers in this subsample were multiparous, I chose to examine parity as a continuous variable. On average, mothers in this subsample had 4.0 (±2.4; 0-10) children prior to the HERO-G cohort infants. Average gestational age was 40 (±1.2) weeks. Female offspring (N=28) represented 56.0% of the subsample and males (N=22) 44.0%. Most infants in this subsample were born in the dry season (N=39; 78.0%). Average age at introduction of non-breast milk foods was the same was the full HERO-G subsample described in Chapter 2 (5.0 ±1.5 months), with 36 infants (72%) exclusively breastfed for less than 6 months. Average household SEP for the full HERO-G cohort are detailed below. PCA calculations were constructed for each sample group (the larger HERO-G Cohort, HERO-G Subsample, and HERO-G Milk Analysis Subset) separately. These baseline characteristics of the subsample are detailed in **Table 3.4.**

Table 3.4. Baseline characteristics for the milk analysis subset (N=50) from the HERO-G subsample

Variable	HERO-G Cohort (N=238)	HERO-G Subsample (N=194)	Milk analysis subset (N=50)
Maternal age, years (SD)	31.0 (±6.9)	32.0 (±6.9)	32.4 (±6.5)
Parity, N (%)			
Primiparous	29 (12.2)	18 (9.3)	2 (4.0)
Multiparous	209 (87.8)	176 (90.7)	48 (96.0)
Infant season of birth, N (%)			
Wet season (Jul-Oct)	84 (35.3)	64 (33.0)	11 (22.0)
Dry season (Nov-Jun)	154 (64.7)	130 (67.0)	39 (78.0)
Infant sex, N (%)			
Male	128 (53.8)	103 (53.1)	22 (44.0)
Female	110 (46.2)	91 (46.9)	28 (56.0)
Household SEP, Mean (SD)	-0.24 (1.9)	-1.03 (1.2)	1.34 (2.7)

Data are reported as mean (SD) or mean (%) values.

Maternal MUAC during pregnancy

Maternal MUAC measurements collected during the third trimester of pregnancy were available from 49 of the 50 mothers included in this milk analysis subset. Average (±SD) MUAC was 26.25 cm (±2.3; range: 21.4-31.7). A total of 7 (14.3%) mothers had MUAC measurements of < 23 cm and were thus categorized as undernourished. **Appendix Table A.2-A.4** show full details of maternal MUAC measurements throughout pregnancy. There were no significant ($P<0.05$) differences between mean third trimester MUAC in the HERO-G milk analysis subset, HERO-G subsample, or the larger HERO-G cohort. Similarly, there was no significant difference between MUAC measurements at 20 (N=24) or 28 (N=50) weeks' gestation at any time point. On average, maternal MUAC in the milk analysis subset increased by 0.3 (±1.68) cm between 20 and 36 weeks' gestation, and 0.09 (±1.28) cm between 28 and 36 weeks.

Milk macronutrient composition

Macronutrients

Milk fat content was greatest at 3 months (4.97 g/dL ±1.9), followed by the 9 (4.66 g/dL ±1.2) and 12 (4.82 g/dL ±1.3) month time points, and was lowest at 6 months (4.08 g/dL ±1.5). Wilcoxon Signed-Ranks Test showed that mean milk fat concentration (±SD) was significantly different based on stage of lactation ($P=0.0124$).

Nonparametric comparisons for each pair using Wilcoxon method showed that fat content was significantly lower at 6 months of lactation compared to that at 3 (P=0.0123), 9 (P=0.0109), and 12 (P=0.0033) months.

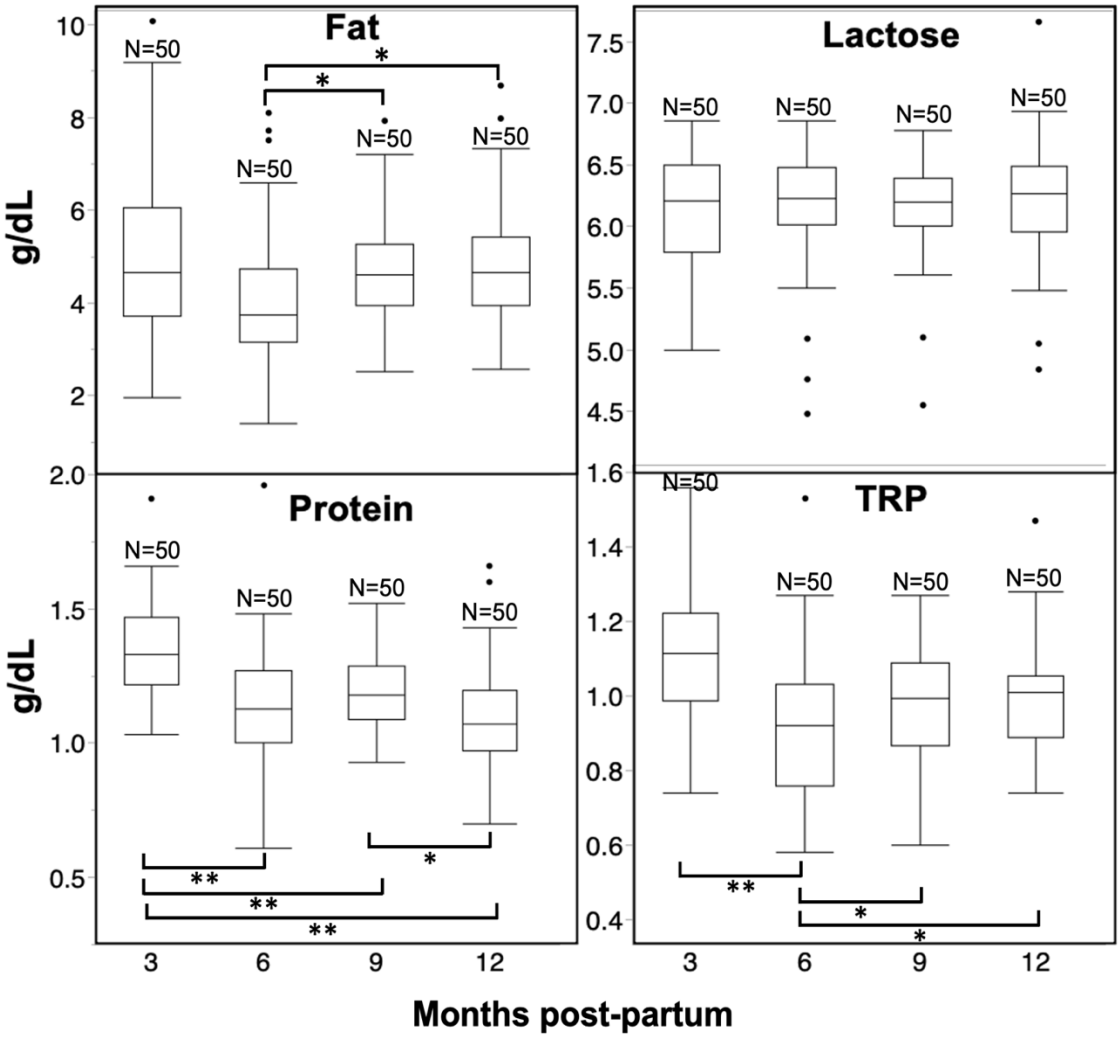
Average protein concentration was 1.35 g/dL (±0.2), 1.14 g/dL (±0.2), 1.20 g/dL (±0.1), and 1.32 g/dL (±0.2) at 3, 6, 9, and 12 months, respectively, showing an initial decrease followed by a positive increase after 6 months of lactation. There was a significant difference in protein concentration based on lactation stage (P<.0001). Protein was significantly higher at 3 months of lactation compared to that at 6 (P<.0001), 9 (P<.0001), and 12 (P<.0001) months.

Average lactose content was similar at 3 (6.15 g/dL ±0.4), 6 (6.11 g/dL ±0.6), and 9 (6.16 g/dL ±0.4) months of lactation, followed by a slight (but non-significant) increase at 12 months of lactation (6.29 g/dL ±0.8). There was no significant (P<0.05) difference in lactose content across the first 12 months of lactation.

The concentration of TRP was highest at 3 months of lactation at 1.10 g/dL (±0.2) and lowest at 6 months (0.91 g/dL ±0.2). At 9 and 12 months, average true protein content increased to 0.97 g/dL (±0.2) and 1.00 g/dL (±0.1), respectively. There was a significant difference in TRP content based on stage of lactation (P<.0001). TRP content at 3 months was significantly higher than concentrations at 6 (P<.0001), 9 (P=0.0006), and 12 (P=0.0014) months of lactation. Additionally, TRP was significantly lower at 6 months relative to 9 (P=0.0455) and 12 (P=0.0065) months of lactation.

Mean values and ranges of milk macronutrient composition (fat, protein, lactose, TRP) at 3, 6, 9, and 12 months post-partum are depicted in **Figure 3.1.**

Figure 3.1. Milk macronutrient composition (g/dL) at 3, 6, 9, and 12 months post-partum



*P<0.05; **P<0.0001; TRP: true protein

Mixed effects model results (macronutrients)

Mixed effects model analyses assessing potential predictors of milk macronutrients across the first 12 months of lactation were conducted separately for fat, protein, lactose, and TRP. All model results are described below and are detailed in **Table 3.5** for each macronutrient.

Fat: Collection time point was a significant predictor of milk fat content. Milk collected at 3 months of lactation significantly predicted higher fat content by 0.35 g/dL (95% CIs: 0.02, 0.67 g/dL; P=0.0382) compared to the referent 12 month collection time point. Milk collected at 6 months significantly predicted lower fat content by -

0.54 g/dL (95% CIs: -0.88, -0.21 g/dL; P= 0.0015) compared to the referent 12 month collection time point. Infant sex was also a significant predictor of milk fat, with milk collected from mothers of female infants predicting significantly higher fat content by 0.33 g/dL (95% CIs: 0.03, 0.62 g/dL; P=0.0305). The random variable, Subject ID, was also a significant predictor of milk fat (P=0.0304).

Protein: Collection time point was also a significant predictor of milk protein in the mixed effects model. Milk collected at 3 months predicted higher protein content by 0.15 g/dL (95% CIs: 0.12, 0.19 g/dL; P=<0.0001), and milk collected at 6 months significantly predicted lower protein content by -0.05 g/dL (95% CIs: -0.08, -0.01 g/dL; P=0.0077) compared to the referent 12 month collection time point. The random variable, Subject ID, was also a significant predictor of milk protein (P=0.0007).

Lactose: The only significant predictor of milk lactose in the mixed effects model was the random variable, Subject ID (P=0.0404).

TRP: Collection time point was a significant predictor of milk TRP content. Milk collected at 3 months of lactation significantly predicted higher TRP by 0.1 g/dL (95% CIs: 0.07, 0.13 g/dL); P<0.0001), and milk collected at 6 months significantly predicted lower TRP content by -0.07 g/dL (95% CIs: -0.11, 0.04 g/dL; P<0.0001) compared to the referent 12 month collection time point. Seasonality was a significant predictor of milk TRP. Milk collected during the dry season significantly predicted higher TRP by 0.02 g/dL (95% CIs: 0, 0.04 g/dL; P=0.0247) relative to milk collected during the wet season. Subject ID was also a significant predictor of TRP (P=0.0009).

Table 3.5. Milk macronutrient mixed effects model results

		Fat			Protein			Lactose			TRP		
	Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Fixed effects	Intercept	4.95	1.55, 8.35	**0.0053***	1.24	0.72, 1.76	**<.0001***	5.54	4.27, 6.81	**<.0001***	0.95	0.5, 1.4	**0.0001***
	Time point[3mo]	0.35	0.02, 0.67	**0.0382***	0.15	0.12, 0.19	**<.0001***	-0.02	-0.14, 0.11	0.8029	0.1	0.07, 0.13	**<.0001***
	Time point[6mo]	-0.54	-0.88, -0.21	**0.0015***	-0.05	-0.08, -0.01	**0.0077***	-0.08	-0.21, 0.04	0.19	-0.07	-0.11, -0.04	**<.0001***
	Time point[9mo]	-0.01	-0.34, 0.31	0.9313	0	-0.04, 0.03	0.9501	-0.02	-0.15, 0.1	0.7264	-0.03	-0.06, 0.01	0.1056
	Time point[12] *Referent*	-	-	-	-	-	-	-	-	-	-	-	-
	Maternal MUAC	0.01	-0.12, 0.13	0.901	0	-0.02, 0.01	0.6425	0.02	-0.03, 0.07	0.3777	0	-0.02, 0.02	0.9761
	SEP Score	-0.09	-0.2, 0.02	0.1114	0.01	-0.01, 0.03	0.2176	0.01	-0.03, 0.05	0.4992	0.01	0, 0.02	0.1842
	CollectionSeason[Dry]	0.05	-0.16, 0.26	0.6348	0.01	-0.01, 0.03	0.4309	-0.03	-0.11, 0.05	0.5119	0.02	0, 0.04	**0.0247***
	EBF duration (mo)	0.01	-0.19, 0.2	0.9541	0.01	-0.02, 0.04	0.6421	0.05	-0.02, 0.12	0.1787	0.01	-0.02, 0.03	0.5801
	Sex[F]	0.33	0.03, 0.62	**0.0305***	-0.01	-0.05, 0.04	0.8029	-0.05	-0.17, 0.06	0.3222	0	-0.04, 0.04	0.9055
	BirthSeason[Dry]	-0.35	-0.72, 0.02	0.0599	0	-0.06, 0.05	0.9715	0.02	-0.12, 0.15	0.8015	-0.03	-0.08, 0.02	0.195
	Parity	-0.07	-0.21, 0.06	0.2834	0.01	-0.01, 0.03	0.4694	-0.04	-0.09, 0.01	0.1121	0	-0.02, 0.02	0.8366
Random effects	SubjectID	0.43	0.04, 0.81	**0.0304***	0.01	0.01, 0.02	**0.0007***	0.06	0, 0.11	**0.0404***	0.01	0, 0.02	**0.0009***

TRP: true protein; Time point: sample collection time point (3, 6, 9, or 12mo); CollectionSeason: season of milk collection [wet/dry]; BirthSeason: infant season of birth [wet/dry]; LB, UB: 95% CIs (Lower Bound, Upper Bound); *P<0.05; Variable significance is indicated by bolded font/gray cell highlight.

Multiple linear regression results (macronutrients)

Milk fat

The multiple linear regression models constructed for the "real time" predictors of milk fat were non-significant (P>0.05), as were the "extended" predictors of milk fat examining conditions at 3 months of lactation as potential predictors of milk fat at 9 and 12 month time points and the model examining conditions at 6 months as potential predictors of milk fat at 9 months of lactation. The model examining conditions at 6 months of lactation as potential predictors of milk fat at 12 months was significant (P=0.0449) and had a large effect size (f^2=0.38). When adjusting for conditions at 6 months, milk fat content at 12 months was significantly predicted by infant sex, with milk collected from mothers of female infants containing more fat by 0.43 g/dL (95% CIs: 0.05, 0.81 g/dL; P=0.0267) compared to mothers of male infants. Milk fat at 12 months was also significantly predicted by EBF status at 6 months,

with milk collected from mothers of infants still receiving only maternal milk in their diet at 6 months of age containing more fat by 0.52 g/dL (95% CIs: 0.11, 0.94 g/dL; P=0.0144) at 12 months. Model results are detailed in **Table 3.6**.

Table 3.6. Results of multiple linear regression models examining "Real time" and "Extended" predictors of milk fat across the first 12 months of lactation

	"Real time" predictors of milk fat											
Time Point	3mo (MS: P=0.4407)			6mo (MS: P=0.1025)			9mo (MS: P=0.7052)			12mo (MS: P=0.257)		
Value	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	7.44	0.82, 14.07	**0.0287***	3.72	-1.37, 8.81	0.1477	5.49	1.37, 9.62	0.0103*	2.83	-1.71, 7.36	0.2153
Sex[F]	0.23	-0.36, 0.82	0.4303	0.55	0.1, 1	0.0187*	0.21	-0.17, 0.58	0.2693	0.42	0.01, 0.82	0.0432*
Maternal MUAC	-0.04	-0.3, 0.22	0.7589	0.03	-0.17, 0.23	0.7809	-0.03	-0.19, 0.13	0.7213	0.08	-0.1, 0.26	0.3966
Parity	-0.23	-0.5, 0.05	0.1038	-0.05	-0.27, 0.16	0.6085	0.03	-0.15, 0.2	0.7669	0	-0.18, 0.19	0.9578
CollectionSeason[Dry]	-0.16	-0.81, 0.5	0.6278	0.43	-0.11, 0.97	0.1146	-0.03	-0.41, 0.35	0.8896	-0.58	-2.07, 0.91	0.4337
BirthSeason[Dry]	-0.78	-1.54, -0.02	0.0444*	-0.07	-0.73, 0.6	0.8439	-0.15	-0.62, 0.32	0.5221	0.37	-1.08, 1.82	0.6079
EBF Status @3mo [Y]	0.16	-0.67, 1	0.6977	-	-	-	-	-	-			
EBF Status @6mo[Y]	-	-	-	-0.08	-0.58, 0.42	0.7484	-	-	-			
SEP Score	-0.11	-0.33, 0.12	0.3444	-0.17	-0.34, 0.01	0.0579	-0.11	-0.25, 0.03	0.1326	0.07	-0.09, 0.23	0.3583
	"Extended" predictors of milk fat											
Time Point	9mo_3mo (MS: P=0.6776)			9mo_6mo (MS: P=0.6717)			12mo_3mo (MS: P=0.3045)			***12mo_6mo (MS: P=0.0449)***		
Value	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	5.1	0.9, 9.3	0.0184*	5.78	1.62, 9.94	0.0076*	2.52	-2.11, 7.15	0.2777	1.95	-2.37, 6.27	0.3675
Sex[F]	0.22	-0.15, 0.6	0.2355	0.21	-0.17, 0.58	0.2706	0.44	0.03, 0.85	0.0366*	0.43	0.05, 0.81	**0.0267***
Maternal MUAC	-0.02	-0.19, 0.14	0.7910	-0.05	-0.22, 0.12	0.5713	0.08	-0.1, 0.26	0.3647	0.13	-0.05, 0.3	0.1424
Parity	0.03	-0.15, 0.2	0.7570	0.05	-0.13, 0.23	0.5896	0.01	-0.18, 0.2	0.9456	-0.05	-0.23, 0.13	0.6062
CollectionSeason[Dry]	0.01	-0.38, 0.39	0.9762	-0.07	-0.46, 0.32	0.7124	-0.68	-2.2, 0.83	0.3690	-0.48	-1.88, 0.93	0.4962
BirthSeason[Dry]	-0.19	-0.67, 0.29	0.4276	-0.11	-0.59, 0.37	0.6569	0.42	-1.04, 1.88	0.5639	0.13	-1.24, 1.51	0.8462
EBF Status @3mo [Y]	0.27	-0.25, 0.79	0.3086	-	-	-	0.23	-0.34, 0.79	0.4286	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.22	-0.63, 0.2	0.2984	-	-	-	0.52	0.11, 0.94	**0.0144***
SEP Score	-0.1	-0.24, 0.05	0.1732	-0.11	-0.26, 0.03	0.1197	0.08	-0.08, 0.24	0.3090	0.07	-0.08, 0.22	0.3405

"Extended" predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value *P<0.01; Model and variable significance are indicated by bolded font/dark gray cell highlight (Note: only variables from significant models were indicated).

Protein

At the 3, 9, and 12 month milk collection time points, the multiple linear regression models constructed for the "real time" predictors of milk protein were non-significant (P>0.05), as were all four models examining potential

"extended" predictors of milk fat using conditions at the 3 and 6 month time points as potential predictors of milk fat concentrations at 9 and 12 month time points. The model assessing potential real time predictors of milk protein at 6 months was significant (P=0.0401) and had a large effect size (f^2=0.35). Milk protein content at 6 months was significantly predicted by season of collection, with milk collected from mothers during the dry season containing more protein by 0.13 g/dL (95% CIs: 0.05, 0.21 g/dL; P=0.0019) compared to milk collected during the wet season. Model results are detailed in **Table 3.7**.

Table 3.7. Results of multiple linear regression models examining “Real time” and “Extended” predictors of milk protein across the first 12 months of lactation

	"Real time" predictors of milk protein											
Time Point	3mo (MS: P=0.9959)			**6mo (MS: P=0.0401)**			9mo (MS: P=0.6649)			12mo (MS: P=0.2387)		
Value	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	1.43	0.75, 2.11	0.0001*	1.23	0.49, 1.97	**0.0017***	1.36	0.88, 1.83	<.0001*	0.81	0.17, 1.44	0.0138*
Sex[F]	0	-0.06, 0.06	0.8974	0.02	-0.05, 0.08	0.5694	0	-0.04, 0.04	0.935	-0.03	-0.09, 0.03	0.2871
Maternal MUAC	0	-0.03, 0.02	0.8198	-0.01	-0.04, 0.02	0.5544	-0.01	-0.03, 0.01	0.4559	0.01	-0.02, 0.03	0.6027
Parity	0	-0.03, 0.03	0.9081	0.01	-0.02, 0.05	0.361	0	-0.02, 0.02	0.6486	0.02	0, 0.05	0.0738
CollectionSeason[Dry]	0	-0.06, 0.07	0.9499	0.13	0.05, 0.21	**0.0019***	-0.02	-0.07, 0.02	0.2563	0.17	-0.04, 0.37	0.1146
BirthSeason[Dry]	-0.02	-0.1, 0.06	0.6345	0.1	0, 0.19	0.0501	0	-0.05, 0.05	0.9976	-0.14	-0.34, 0.06	0.1723
EBF Status @3mo [Y]	0.01	-0.07, 0.1	0.7575	-	-	-	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.02	-0.09, 0.06	0.6561	-	-	-	-	-	-
SEP Score	0.01	-0.02, 0.03	0.59	0.02	-0.01, 0.04	0.1329	0.01	-0.01, 0.03	0.2851	0.01	-0.01, 0.03	0.4308
	"Extended" predictors of milk protein											
Time Point	9mo_3mo (MS: P=0.6895)			9mo_6mo (MS: P=0.7571)			12mo_3mo (MS: P=0.3188)			12mo_6mo (MS: P=0.3406)		
Value	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	1.37	0.88, 1.86	<.0001*	1.37	0.89, 1.85	<.0001*	0.78	0.13, 1.43	0.0198*	0.57	0.1, 1.04	0.0196*
Sex[F]	0	-0.04, 0.05	0.9508	0	-0.04, 0.05	0.9369	-0.03	-0.09, 0.03	0.3252	0.01	-0.04, 0.05	0.7676
Maternal MUAC	-0.01	-0.03, 0.01	0.4513	-0.01	-0.03, 0.01	0.4235	0.01	-0.02, 0.03	0.5788	0.01	0, 0.03	0.1299
Parity	0	-0.02, 0.02	0.6543	0.01	-0.02, 0.03	0.5965	0.02	0, 0.05	0.0751	0.01	-0.01, 0.03	0.2632
CollectionSeason[Dry]	-0.03	-0.07, 0.02	0.2534	-0.03	-0.07, 0.02	0.2385	0.16	-0.06, 0.37	0.1433	0.15	-0.01, 0.3	0.0623
BirthSeason[Dry]	0	-0.05, 0.06	0.97	0	-0.05, 0.06	0.9431	-0.13	-0.34, 0.07	0.1916	-0.14	-0.29, 0.01	0.0718
EBF Status @3mo [Y]	-0.01	-0.07, 0.05	0.8271	-	-	-	0.02	-0.06, 0.1	0.6208	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.01	-0.06, 0.04	0.7026	-	-	-	0.02	-0.02, 0.07	0.3451
SEP Score	0.01	-0.01, 0.03	0.3081	0.01	-0.01, 0.03	0.2999	0.01	-0.01, 0.03	0.3993	0.01	-0.01, 0.02	0.355

“Extended” predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound)P: P-value *P<0.01; Model and variable significance are indicated by bolded font/dark gray cell highlight (Note: only variables from significant models were indicated).

Lactose

None of the multiple linear regression models were significant (P>0.05) predictors of milk lactose content at any time point across the first 12 months of lactation using either real time or extended predictors. Results from the statistical models are detailed in **Table 3.8**.

Table 3.8. Results of multiple linear regression models examining "Real time" and "Extended" predictors of milk lactose across the first 12 months of lactation

	"Real time" predictors of milk lactose											
Milk Collection Time point	3mo (MS: P=0.2604)			6mo (MS: P=0.2868)			9mo (MS: P=0.6138)			12mo (MS: P=0.3016)		
Value	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	5.35	3.92, 6.77	<.0001*	6.31	4.34, 8.27	<.0001*	6.31	4.98, 7.64	<.0001*	5.64	2.89, 8.38	0.0002*
Sex[F]	-0.1	-0.23, 0.02	0.1066	0.02	-0.15, 0.2	0.8164	-0.02	-0.14, 0.1	0.7135	-0.21	-0.45, 0.04	0.0948
Maternal MUAC	0.03	-0.03, 0.08	0.3081	0.01	-0.07, 0.09	0.835	0	-0.05, 0.05	0.9804	0.04	-0.07, 0.15	0.4726
Parity	0.03	-0.03, 0.09	0.319	-0.07	-0.15, 0.02	0.1077	-0.04	-0.1, 0.01	0.1402	-0.1	-0.22, 0.01	0.0794
CollectionSeason[Dry]	0.02	-0.12, 0.16	0.8044	-0.17	-0.38, 0.03	0.098	-0.03	-0.15, 0.1	0.6546	-0.06	-0.96, 0.84	0.8945
BirthSeason[Dry]	0.11	-0.06, 0.27	0.1933	-0.15	-0.41, 0.1	0.2295	0.02	-0.13, 0.17	0.7939	0.1	-0.77, 0.98	0.8104
EBF Status @3mo [Y]	-0.14	-0.32, 0.04	0.1187	-	-	-	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	0.13	-0.06, 0.33	0.1823	-	-	-	-	-	-
SEP Score	0	-0.05, 0.05	0.9225	0	-0.07, 0.06	0.9201	0.01	-0.04, 0.05	0.8084	0.04	-0.05, 0.14	0.3723
	"Extended" predictors of milk lactose											
Milk Collection Time point_Time point Conditions	9mo_3mo (MS: P=0.5336)			9mo_6mo (MS: P=0.7172)			9mo_3mo (MS: P=0.3021)			12mo_6mo (MS: P=0.284)		
Value	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	1.15	0.55, 1.75	0.0004*	5.37	2.6, 8.15	0.0003*	5.38	2.6, 8.17	0.0003*	5.37	2.6, 8.15	0.0003*
Sex[F]	-0.03	-0.09, 0.02	0.218	-0.2	-0.45, 0.04	0.1008	-0.19	-0.44, 0.06	0.1266	-0.2	-0.45, 0.04	0.1008
Maternal MUAC	-0.01	-0.03, 0.02	0.5885	0.05	-0.06, 0.17	0.3281	0.04	-0.06, 0.15	0.42	0.05	-0.06, 0.17	0.3281
Parity	0	-0.03, 0.02	0.7742	-0.12	-0.23, 0	0.0493*	-0.1	-0.21, 0.01	0.0825	-0.12	-0.23, 0	0.0493*
CollectionSeason[Dry]	-0.01	-0.07, 0.04	0.6885	-0.03	-0.93, 0.87	0.95	-0.14	-1.05, 0.77	0.757	-0.03	-0.93, 0.87	0.95
BirthSeason[Dry]	-0.03	-0.1, 0.04	0.4243	0.03	-0.85, 0.92	0.9378	0.15	-0.73, 1.02	0.7396	0.03	-0.85, 0.92	0.9378
EBF Status @3mo [Y]	0.01	-0.07, 0.08	0.8926	-	-	-	0.18	-0.16, 0.53	0.2838	-	-	-
EBF Status @6mo[Y]	-	-	-	0.16	-0.11, 0.42	0.2438	-	-	-	0.16	-0.11, 0.42	0.2438
SEP Score	0.01	-0.01, 0.04	0.1538	0.04	-0.05, 0.14	0.3759	0.05	-0.05, 0.15	0.2997	0.04	-0.05, 0.14	0.3759

"Extended" predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value; Model and variable significance are indicated by bolded font/dark gray cell highlight (Note: only variables from significant models were indicated).

True protein (TRP)

The multiple linear regression models constructed for the "real time" predictors of milk TRP were non-significant (P>0.05) at 3, 9, and 12 month time points, as were the models examining "extended" predictors of milk TRP at 9 and 12 month point using dietary variables at 6 months, and TRP at 9 months using dietary variables at 3 months. The model examining conditions at 6 months as potential predictors of milk TRP was significant (P=0.0123) and had a large effect size (f^2=0.51). At 6 months of age, seasonality predicted milk TRP content, with milk collected during the dry season predicting higher TPR by 0.11 g/dL (95% CIs: 0.05, 0.17 g/dL; P=0.0007). The model examining potential predictors of milk TRP at 12 months under the conditions at 3 months of age was significant (P=0.0448) and had a large effect size (f^2=0.38). When adjusting for conditions at 3 months, milk TRP content at 12 months was significantly predicted by EBF status at 3 months, with milk collected from mothers of infants who were still EBF at 3 months containing more TRP by 0.06 g/dL (95% CIs: 0.01, 0.12 g\/dL; P=0.0280) compared to mothers of infants who were receiving non-breast milk foods at 3 months of age. Full model results are detailed in **Table 3.9**.

Table 3.9. Results of multiple linear regression models examining "Real time" and "Extended" predictors of milk TRP across the first 12 months of lactation

	"Real time" predictors of milk TRP											
Time point	3mo (MS: P=0.7621)			**6mo (MS: P=0.0123*)**			9mo (MS: P=0.4115)			12mo (MS: P=0.1732)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	1.09	0.49, 1.7	0.0007*	0.91	0.33, 1.48	**0.0028***	1.16	0.58, 1.74	0.0002*	0.6	0.14, 1.07	0.0122*
Sex[F]	0	-0.05, 0.06	0.886	0.02	-0.03, 0.07	0.4447	-0.03	-0.09, 0.02	0.2068	0.01	-0.04, 0.05	0.7904
Maternal MUAC	0	-0.02, 0.02	0.9402	0	-0.03, 0.02	0.8121	-0.01	-0.03, 0.02	0.5743	0.01	-0.01, 0.03	0.1817
Parity	0	-0.03, 0.02	0.8382	0.01	-0.01, 0.03	0.3931	0	-0.03, 0.02	0.7703	0.01	-0.01, 0.03	0.173
CollectionSeason[Dry]	0.02	-0.04, 0.07	0.5977	0.11	0.05, 0.17	**0.0007***	-0.01	-0.07, 0.04	0.6645	0.14	-0.01, 0.29	0.0692
BirthSeason[Dry]	-0.05	-0.12, 0.02	0.1789	0.03	-0.04, 0.11	0.3533	-0.03	-0.09, 0.04	0.4253	-0.13	-0.28, 0.02	0.09
EBF Status @3mo [Y]	0.01	-0.07, 0.08	0.8485	-	-	-	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.03	-0.08, 0.03	0.3675	-	-	-	-	-	-
SEP Score	0.01	-0.01, 0.03	0.3892	0.01	-0.01, 0.03	0.4116	0.01	-0.01, 0.03	0.1504	0.01	-0.01, 0.02	0.3499
	"Extended" predictors of milk TRP											
Time point	9mo_3mo (MS: P=0.5336)			9mo_6mo (MS: P=0.433)			**12mo_3mo (MS: P=0.0448*)**			12mo_6mo (MS: P=0.1969)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	1.15	0.55, 1.75	0.0004*	1.2	0.61, 1.78	0.0002*	0.52	0.07, 0.97	**0.0252***	0.57	0.1, 1.04	0.0196*
Sex[F]	-0.03	-0.09, 0.02	0.218	-0.03	-0.09, 0.02	0.2062	0.01	-0.03, 0.05	0.5716	0.01	-0.04, 0.05	0.7676
Maternal MUAC	-0.01	-0.03, 0.02	0.5885	-0.01	-0.03, 0.01	0.4584	0.01	0, 0.3	0.1151	0.01	0, 0.03	0.1299
Parity	0	-0.03, 0.02	0.7742	0	-0.03, 0.02	0.9538	0.01	0, 0.03	0.1418	0.01	-0.01, 0.03	0.2632
CollectionSeason[Dry]	-0.01	-0.07, 0.04	0.6885	-0.02	-0.07, 0.04	0.5289	0.11	-0.03, 0.26	0.1283	0.15	-0.01, 0.3	0.0623
BirthSeason[Dry]	-0.03	-0.1, 0.04	0.4243	-0.02	-0.09, 0.05	0.5371	-0.11	-0.26, 0.03	0.1136	-0.14	-0.29, 0.01	0.0718
EBF Status @3mo [Y]	0.01	-0.07, 0.08	0.8926	-	-	-	0.06	0.01, 0.12	**0.0280***	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.03	-0.09, 0.03	0.3523	-	-	-	0.02	-0.02, 0.07	0.3451
SEP Score	0.01	-0.01, 0.04	0.1538	0.01	-0.01, 0.03	0.1656	0.01	-0.01, 0.03	0.1981	0.01	-0.01, 0.02	0.355

"Extended" predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value *P<0.01; Model and variable significance are indicated by bolded font/dark gray cell highlight (Note: only variables from significant models were indicated).

A post-hoc Wilcoxon Signed-Ranks Test was conducted to further investigate seasonal differences in maternal milk TRP composition based on the month of milk production across the first 12 months of lactation. TRP composition was significantly different (P=0.0230) based on month of milk collection at the 6 month collection time point, but not at 3, 9, or 12 month time points. TRP content at 6 months is detailed according to month of milk production in **Table 3.10.** At 6 months, maternal milk collected during the month of March (N=3) contained significantly more TRP than milk collected during July (Z = 0.36, P = 0.0006), August (Z = 0.26, P = 0.0112), September (Z = ; P = 0.0030), October (Z = -2.7; P = 0.0077) – which encapsulates all of the months in the wet ("hungry") season – and December (Z = -2.0; P = 0.0464). Milk produced in July (Z = -2.4; P = 0.0153) and September (Z = -2.5; P =0.0137) contained significantly lower concentrations of TRP compared to milk produced in January (Z = -2.4; P = 0.0153 and Z = -2.5; P =0.0137, respectively) (**Table 3.11**).

Table 3.10. TRP content according to month of milk collection

Level	N	Mean	Std Err Mean	LB, UB
Jan	1	1 (.)	-	-
Feb	4	1.01 (0.03)	0.02	0.96, 1.06
Mar	3	1.13 (0.14)	0.08	0.79, 1.47
Apr	4	0.89 (0.2)	0.10	0.58, 1.20
May	3	1.01 (0.13)	0.08	0.69, 1.34
Jun	3	1.05 (0.06)	0.04	0.89, 1.21
Jul	9	0.77 (0.11)	0.04	0.69, 0.86
Aug	7	0.87 (0.11)	0.04	0.76, 0.97
Sep	5	0.82 (0.24)	0.11	0.53, 1.12
Oct	5	0.83 (0.15)	0.07	0.64, 1.01
Nov	4	0.97 (0.16)	0.08	0.70, 1.23
Dec	1	0.93 (.)	-	-

LB, UB: 95% CIs (Lower Bound, Upper Bound)

Table 3.11. Wilcoxon Signed-Ranks Test results comparing milk TRP by month of milk production

Level	- Level	Difference	Std Err Dif	P	LB, UB
Feb	Jul	0.23	0.09	**0.0100***	0.06, 0.41
Mar	Jul	0.36	0.1	**0.0006****	0.16, 0.55
Mar	Sep	0.31	0.1	**0.0056***	0.1, 0.52
Mar	Oct	0.30	0.1	**0.0065***	0.09, 0.51
Mar	Aug	0.26	0.1	**0.0112***	0.06, 0.46
Mar	Apr	0.24	0.11	**0.0331***	0.02, 0.46
May	Jul	0.24	0.1	**0.0166***	0.05, 0.43
Jun	Sep	0.23	0.1	**0.0360***	0.02, 0.44
Jun	Oct	0.22	0.1	**0.0409***	0.01, 0.43
Nov	Jul	0.19	0.09	**0.0324***	0.02, 0.37

LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value; *P<0.05; **P<0.01; Months not mentioned in the Table had non-significant differences

Relative abundance of maternal milk HMO classes

Samples and power analysis

Results were generated for relative abundance of HMO classes in milk samples collected at 3 (N=28), 6 (N=45), 9 (N=46), and 12 (N=50) months post-partum, for a total of N=169 samples from the original design of N=200 (*Note:* the reduced sample size is attributed to insufficient sample volumes). A total of 26 mothers had milk samples that were analyzed at all four time points, 20 mothers had milk analyzed from three time points, 2 mothers had milk analyzed from two time points, and 2 mothers had milk analyzed from only one time point (**Table 3.12**).

Table 3.12. Maximum number of samples analyzed per mother (N=50) per time point in the milk analysis subset

# time points analyzed	**1**	**2**	**3**	**4**	*Total*
# mothers	2	2	20	26	50

A post-hoc statistical power analysis (G*Power Version 3.1) showed that for a medium effect size (f^2=0.15) for a linear multiple regression with a significance level of $\alpha = 0.05$ to examine predictors of each milk constituent of interest by collection time point, a total sample size of 50 has a power of 0.89. Under the same effect size and

significance level, a sample size of 28 – the number of observations available from 3 months of lactation – has a power of 0.77.

Secretor status

Of the 50 mothers included in this subsample, 38 (76%) were Secretors and 12 (24%) were non-Secretors. As previously described, mothers with less than 6% relative α(1-2) fucosylation (2'FL) were assigned as phenotypic non-Secretors; assigned Secretor status was consistent at all time points except in three samples from milk collected at 6 months and one from 9 months post-partum. The shifts in phenotypic secretor status across the first 12 months of lactation are detailed in **Table 3.13.**

Table 3.13. Shifts in maternal Secretor status based on maternal milk phenotype in 4 study participants

Collection time point / ***Sample ID***	**3**	**6**	**9**	**12**
Individual 1	NS	S	NS	NS
Individual 2	S	NS	S	S
Individual 3	Sample not analyzed	NS	S	NS
Individual 4	S	NS	S	S

S: Secretor; NS: non-Secretor

For Individual 1 and 2, milk collected at 6 months post-partum (the time point with a different Secretor phenotype than the other time points) were both collected in the month of September (wet season) but in different years. For Individuals 3 and 4, the milk samples with a different Secretor phenotype compared to all other milk samples (9 month sample and 6 month sample for Individual 3 and 4, respectively) were both collected in September of the same year, but on different dates. None of the four mothers were classified as underweight (MUAC < 23 cm) during pregnancy. All were multiparous having 1-4 prior live births. Two were mothers of male infants and two of female infants. There were no significant differences in any of the baseline population characteristics based on Secretor status. Age at which complementary foods were introduced was similar between Secretor and non-Secretor mothers (**Table 3.14).**

Table 3.14. Infant feeding practices in the milk analysis subset and according to Secretor status

Variable	Milk analysis subset (N=50)	Secretor (N=38)	Non-Secretor (N=12)
EBF duration, N (%)			
<6mo	36 (72.0)	14 (70.0)	6 (75.0)
≥6mo	14 (28.0)	6 (30.0)	2 (25.0)
Average (mo)	5.0 (1.5)	5.0 (1.4)	5.0 (1.8)

Data are reported as mean (SD) or mean (%) values.

HMO classes

Mean (SD) relative abundances of fucosylated, sialylated, undecorated, and sia-fuc HMO classes across the first 12 months of are provided in **Table 3.15.** Relative abundances of fucosylated HMOs increased over the lactation period for Secretors (from 54.67% at 3 months to 65.10% at 12 months). For non-Secretors, average fucosylated HMO relative abundance ranged from 50.94 to 54.74% across the first 12 months of lactation (**Figure 3.2**). Non-Secretor mothers had a wider range of relative abundance of fucosylated HMOs compared to Secretors between the 3 and 6 month time point (non-significant) for Secretors but remained nearly identical between those time points for non-Secretors.

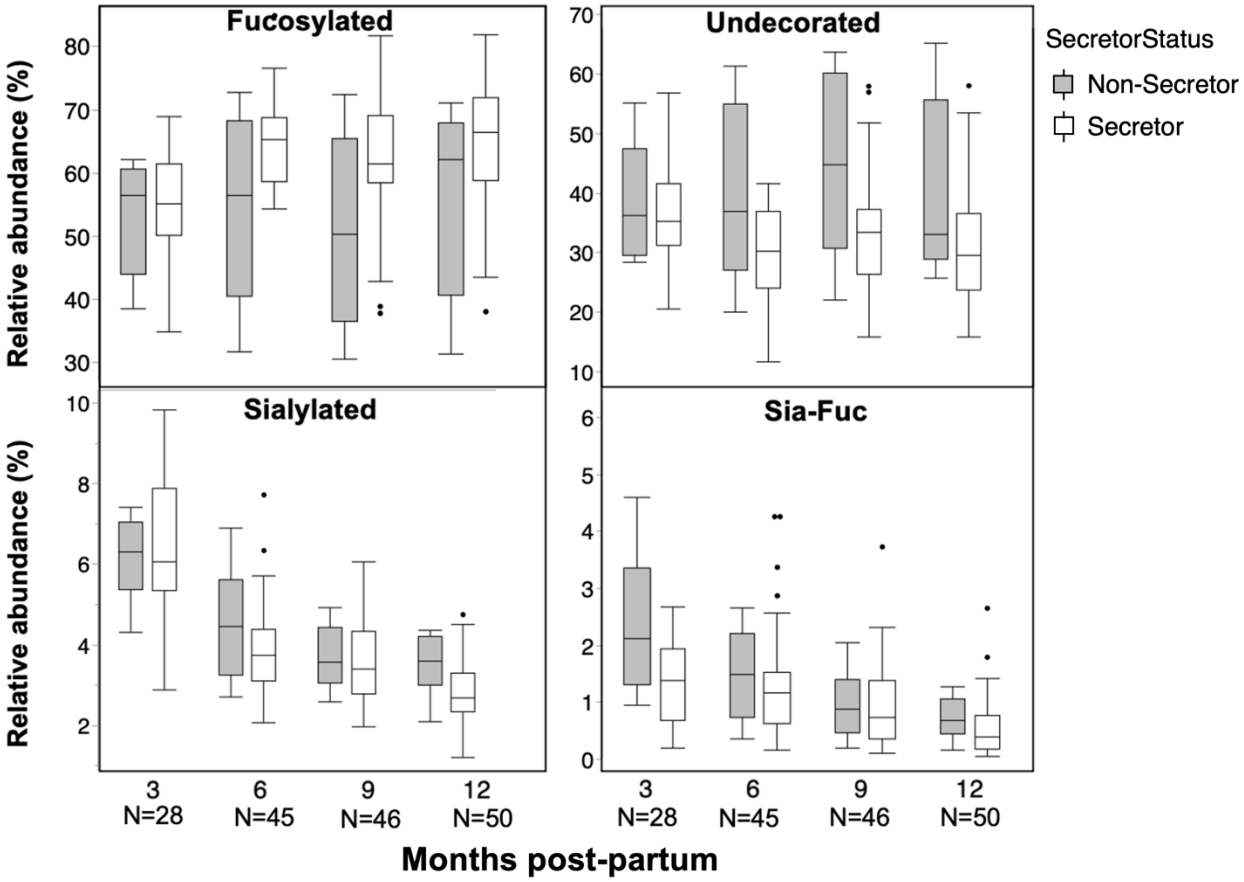
Relative abundances of all HMO classes were significantly different between Secretor and non-Secretor mothers; relative abundance of fucosylated HMOs was significantly higher in Secretors (P=0.0006), while milk from non-Secretor mothers contained significantly greater relative abundances of sialylated (P=0.0258) and undecorated (P=0.0124) HMO classes. The average relative abundance of 2'FL, the fucosylated HMO structure used to determine Secretor status, was 8.88% in Secretor mothers.

Table 3.15. Milk HMO composition at 3, 6, 9, and 12 months post-partum

Age (mo)	Se status	N	Fucosylated		Sialylated		Undecorated		Sia-Fuc	
			Mean %	*SD*	*Mean %*	*SD*	*Mean %*	*SD*	*Mean %*	*SD*
3	**NS**	8	53.5	9.11	6.16	1.04	37.91	10.01	2.42	1.26
	S	20	54.67	8.55	6.40	1.74	37.03	9.06	1.60	1.55
6	**NS**	13	54.34	14.3	4.54	1.35	39.62	14.22	1.49	0.78
	S	32	64.51	6.98	3.92	1.25	30.21	7.28	1.36	1.07
9	**NS**	10	50.94	15.45	3.66	0.74	44.44	15.59	0.96	0.57
	S	36	62.30	9.85	3.60	1.14	33.19	9.50	0.91	0.79
12	**NS**	12	54.74	15.16	3.50	0.71	42.04	14.93	0.71	0.37
	S	38	65.10	10.38	2.88	0.82	31.46	10.21	0.56	0.54

Se status: Secretor status; NS: Non-Secretor; S: Secretor

Figure 3.2. Relative abundances (%) of HMO classes at 3, 6, 9, and 12 months post-partum



Mixed effects model results (HMO classes)

After adjusting for maternal nutritional status during pregnancy, maternal parity, EBF duration, infant sex, maternal Secretor status, household SEP, and seasonality, the only significant predictors of relative abundance of the 4 HMO classes were milk collection time point and the random variable, Subject ID. Full model results are described below and detailed in **Table 3.16.**

Collection time point (a variable used to control for lactation stage) was a significant predictor of relative abundance of fucosylated HMO in the HERO-G milk analysis subset. Milk collected at 3 months of lactation significantly predicted lower relative abundance of fucosylated HMO (B: -5.95%; 95% CIs: -7.84, -4.06%; P<0.0001), higher relative abundance of sialylated HMO (B: 2.06%; 95% CIs: 1.79, 2.33%; P<0.0001), higher relative abundance of undecorated HMO (B: 3.21%; 95% CIs: 1.21, 5.20%; P=0.0019), and higher relative abundance of sia-fuc HMO (B: 0.67%; 95% CIs: 0.46, 0.88%; P<0.0001). Milk collected at 6 months of lactation significantly predicted higher relative abundance of fucosylated HMO (1.92%; 95% CIs: 0.34, 3.49%; P=0.0174), lower undecorated HMO (B:-2.0%; 95% CIs: -3.66, -0.34%; P=0.0185), and higher sia-fuc HMO (B: 0.67%; 95% CIs: 0.46, 0.88%; P<0.0001). Milk collected at 9 months of lactation significantly predicted lower sialylated HMO (B: -0.64%; 95% CIs: -0.86, -0.42%; P<0.0001) and sia-fuc HMO (B: -0.28%; 95% CIs: -0.45, -0.12%; P=0.0011).

The random variable, Subject ID, was also a significant predictor of higher relative abundance of fucosylated HMO (106.41%; 95% CIs: 51.78, 161.4%; P=0.0001), sialylated HMO (B: 0.65%; 95% CIs: 0.28, 1.01%; P=0.0005), undecorated HMO (B: 100.74%; 95% CIs: 48.03, 153.45%; P=0.0002), and sia-fuc HMO (B: 0.48%; 95% CIs: 0.22, 0.74%; P=0.0003).

Table 3.16. Mixed effects model results (HMO)

		Fuc			Sia			Undec			Sia-Fuc		
	Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Fixed	Intercept	66.59	27.05, 106.14	**0.0016***	6.04	2.68, 9.4	**0.0008***	25.5	-13.26, 64.27	0.1903	2.01	-0.8, 4.82	0.1570
	CollectionTime point[3]	-5.95	-7.84, -4.06	**<.0001***	2.06	1.79, 2.33	**<.0001***	3.21	1.21, 5.2	**0.0019***	0.67	0.46, 0.88	**<.0001***
	CollectionTime point[6]	1.92	0.34, 3.49	**0.0174***	-0.14	-0.37, 0.08	0.2083	-2	-3.66, -0.34	**0.0185***	0.22	0.04, 0.39	**0.0143***
	CollectionTime point[9]	0.38	-1.14, 1.9	0.6223	-0.64	-0.86, -0.42	**<.0001***	0.56	-1.04, 2.17	0.4893	-0.28	-0.45, -0.12	**0.0011***
	CollectionTime point[12] *Referent*	-	-	-	-	-	-	-	-	-	-	-	-
	Maternal MUAC	-0.17	-1.64, 1.31	0.8207	-0.07	-0.19, 0.06	0.2794	0.27	-1.18, 1.72	0.7106	-0.03	-0.14, 0.08	0.5692
	SEP Score	-0.36	-1.64, 0.91	0.5674	-0.01	-0.11, 0.1	0.9076	0.39	-0.86, 1.64	0.5348	-0.01	-0.11, 0.08	0.7525
	CollectionSeason[Dry]	-0.36	-1.36, 0.64	0.4793	0.07	-0.08, 0.21	0.3694	0.28	-0.78, 1.34	0.6034	0.02	-0.09, 0.13	0.6944
	EBF Duration (mo)	0.43	-1.84, 2.7	0.7025	-0.04	-0.23, 0.16	0.6935	-0.35	-2.58, 1.87	0.7494	-0.04	-0.2, 0.13	0.6587
	Sex[F]	-0.44	-3.9, 3.01	0.7959	0.23	-0.06, 0.53	0.1213	0.3	-3.09, 3.69	0.8577	-0.06	-0.31, 0.19	0.6249
	BirthSeason[Dry]	-0.2	-4.49, 4.08	0.9243	-0.19	-0.56, 0.17	0.2901	0.46	-3.74, 4.66	0.8253	-0.04	-0.35, 0.26	0.7690
	Parity	-1.15	-2.73, 0.43	0.1491	0.09	-0.05, 0.22	0.1963	0.97	-0.58, 2.52	0.2105	0.06	-0.05, 0.17	0.3019
	SecretorStatus[Non-Secretor]	0.07	-2.5, 2.65	0.9563	0.13	-0.15, 0.41	0.3576	0.01	-2.62, 2.65	0.9928	0.09	-0.13, 0.32	0.4133
Random	SubjectID	106.41	51.78, 161.04	**0.0001***	0.65	0.28, 1.01	**0.0005***	100.74	48.03, 153.45	**0.0002***	0.48	0.22, 0.74	**0.0003***

B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value; *P<.05; Collection season: Season of milk collection (Dry vs Wet)

Multiple linear regression model results (HMO classes)

Fucosylated HMO

None of the multiple linear regression models were significant (P>0.05) in predicting relative abundance of fucosylated HMO at any time point across the first 12 months of lactation using either “real time” or “extended” predictors. Results from the statistical models are detailed in **Table 3.17.**

Table 3.17. Results of multiple linear regression models examining “Real time” and “Extended” predictors of fucosylated HMO relative abundance across the first 12 months of lactation

	"Real time" predictors of fucosylated HMO											
Time point	3mo (MS: P=0.7884)			6mo (MS: P=0.1621)			9mo (MS: P=0.2257)			12mo (MS: P=0.2689)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	44.6	-0.91, 90.12	0.0543	74.15	37.28, 111.01	0.0002*	65.34	23.02, 107.67	0.0034*	73.42	30.77, 116.08	0.0012*
Sex[F]	-0.83	-5.45, 3.8	0.7114	-1.55	-5.01, 1.91	0.3683	-1.39	-5.28, 2.5	0.4736	-0.25	-4.1, 3.59	0.8942
Maternal MUAC	0.17	-1.75, 2.08	0.8561	-0.49	-1.94, 0.97	0.5004	-0.14	-1.78, 1.51	0.8683	-0.33	-2.01, 1.36	0.6973
Parity	0.94	-1.47, 3.36	0.4236	-0.29	-1.86, 1.27	0.7078	-1	-2.83, 0.82	0.2732	-0.91	-2.72, 0.89	0.3131
CollectionSeason[Dry]	0.03	-4.49, 4.54	0.9903	-3.23	-7.41, 0.96	0.1265	-0.41	-4.43, 3.6	0.836	4.64	-10, 19.29	0.5256
BirthSeason[Dry]	-0.13	-5.82, 5.55	0.9608	-2.12	-7.17, 2.94	0.4015	0.14	-5.09, 5.37	0.9569	-5.34	-19.58, 8.91	0.4538
EBF Status @3mo [Y]	3.16	-2.67, 9	0.2697	-	-	-	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-2	-6.32, 2.32	0.3536	-	-	-	-	-	-
SEP Score	-0.48	-1.98, 1.03	0.5156	-0.38	-1.64, 0.88	0.5453	-0.55	-2.03, 0.93	0.4535	-0.85	-2.36, 0.65	0.2586
SecretorStatus[Non-Secretor]	-1.09	-5.48, 3.29	0.6066	-5.36	-9.08, -1.63	0.0061*	-5.27	-9.79, -0.76	0.0234*	-5.31	-9.8, -0.81	0.0218*
	"Extended" predictors of fucosylated HMO											
Time Point	9mo_3mo (MS: P=0.2189)			9mo_6mo (MS: P=0.3184)			12mo_3mo (MS: P=0.3224)			12mo_6mo (MS: P=0.3536)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	58.3	14.39, 102.21	0.0107*	64.97	21.7, 108.23	0.0043*	70.85	27.34, 114.37	0.0021*	74.81	31.19, 118.43	0.0013*
Sex[F]	-1.38	-5.26, 2.49	0.4739	-1.4	-5.35, 2.55	0.4766	-0.06	-3.96, 3.85	0.9772	-0.31	-4.2, 3.58	0.8742
Maternal MUAC	0.01	-1.65, 1.66	0.9945	-0.11	-1.82, 1.6	0.8981	-0.27	-1.97, 1.43	0.747	-0.42	-2.17, 1.34	0.6325
Parity	-0.87	-2.71, 0.97	0.3437	-1.03	-2.94, 0.87	0.278	-0.91	-2.73, 0.9	0.3158	-0.81	-2.7, 1.09	0.3942
CollectionSeason[Dry]	-0.09	-4.12, 3.95	0.9656	-0.37	-4.48, 3.73	0.8548	3.64	-11.37, 18.64	0.627	4.66	-10.14, 19.47	0.5281
BirthSeason[Dry]	0.08	-5.13, 5.28	0.9769	0.06	-5.36, 5.48	0.9819	-4.76	-19.19, 9.67	0.5088	-5.12	-19.56, 9.32	0.4777
EBF Status @3mo [Y]	3.2	-2.36, 8.76	0.2512	-	-	-	1.94	-3.43, 7.32	0.4693	-	-	-
EBF Status @6mo[Y]	-	-	-	0.31	-4.06, 4.68	0.8856	-	-	-	-0.9	-5.16, 3.36	0.6718
SEP Score	-0.35	-1.86, 1.16	0.6423	-0.55	-2.05, 0.95	0.4638	-0.77	-2.3, 0.76	0.3164	-0.86	-2.38, 0.66	0.2615
SecretorStatus[Non-Secretor]	-5.46	-9.98, -0.95	0.0191*	-5.23	-9.85, -0.61	0.0275*	-5.15	-9.7, -0.61	0.0272*	-5.51	-10.16, -0.87	0.0212*

“Extended” predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value

Sialylated HMO

The multiple linear models assessing potential predictors of relative abundance of sialylated HMO were non-significant at 3, 6, and 9 month time points using "real time" effects, and non-significant for "extended effects" using 3 and 6 months dietary variables (EBF duration) as potential predictors of fucosylated HMO relative abundance at 9 months. The model examining potential predictors of relative abundance at 12 months of lactation was significant (P=0.0296) with a large effect size (f^2=0.43). Milk collected at 12 months of lactation was significantly predicted by maternal parity, with greater parity (by 1 offspring) predicting higher relative abundance of fucosylated HMO by 0.15% (95% CIs: 0.04, 0.27%; P=0.0093). The models constructed to assess predictors of relative abundance of fucosylated HMO at 12 months using conditions at 3 and 6 months of age were both significant (P=0.0473 and P=0.0403, respectively), and both had large effect sizes (f^2=0.44 and f^2=0.45, respectively). Parity was a significant predictor for both of these extended effects models, with higher parity (by 1 offspring) predicting higher relative abundance by 0.15% (95% CIs: 0.04, 0.27%; P=0.0100) and 0.16% (95% CIs: 0.04, 0.28%; P=0.0092), respectively. Model results are detailed in **Table 3.18**.

Table 3.18. Results of multiple linear regression models examining "Real time" and "Extended" predictors of sialylated HMO relative abundance across the first 12 months of lactation

	"Real time" predictors of sialylated HMO											
Time point	3mo (MS: P=0.9504)			6mo (MS: P=0.1906)			9mo (MS: P=0.6207)			**12mo (MS: P=0.0296*)**		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	6.86	-1.87, 15.59	0.1162	6.67	2.14, 11.21	0.0051*	5.63	1.71, 9.54	0.0061*	3.82	1.14, 6.49	**0.0062***
Sex[F]	-0.01	-0.89, 0.88	0.9882	0.45	0.03, 0.88	0.0374*	0.25	-0.11, 0.61	0.1734	0.13	-0.12, 0.37	0.2998
Maternal MUAC	0.01	-0.35, 0.38	0.935	-0.11	-0.28, 0.07	0.2384	-0.09	-0.24, 0.06	0.2274	-0.05	-0.15, 0.06	0.3662
Parity	-0.13	-0.6, 0.33	0.548	0.1	-0.1, 0.29	0.3218	0.11	-0.06, 0.27	0.2164	0.15	0.04, 0.27	**0.0093***
CollectionSeason[Dry]	0.25	-0.61, 1.12	0.5454	0.26	-0.26, 0.77	0.3136	0.01	-0.36, 0.38	0.9492	0.03	-0.89, 0.94	0.9536
BirthSeason[Dry]	-0.2	-1.29, 0.89	0.6982	-0.23	-0.85, 0.39	0.4613	-0.12	-0.61, 0.36	0.6058	-0.12	-1.01, 0.77	0.7905
EBF Status @3mo [Y]	-0.55	-1.67, 0.57	0.3134	0	-0.53, 0.53	0.9936	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-	-	-	-	-	-	-	-	-
SEP Score	-0.02	-0.31, 0.27	0.8672	0.02	-0.14, 0.17	0.844	0.02	-0.12, 0.16	0.779	-0.01	-0.1, 0.09	0.8544
SecretorStatus[Non-Secretor]	-0.15	-1, 0.69	0.7051	0.27	-0.19, 0.73	0.2391	-0.01	-0.43, 0.41	0.9671	0.21	-0.07, 0.49	0.1439
	"Extended" predictors of sialylated HMO											
Time point	9mo_3mo (MS: P=0.6551)			9mo_6mo (MS: P=0.7297)			**12mo_3mo (MS: P=0.0473*)**			**12mo_6mo (MS: P=0.0403*)**		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	6.07	1.97, 10.18	0.0048*	5.64	1.64, 9.65	0.0070*	3.94	1.2, 6.67	**0.0059***	3.92	1.18, 6.65	**0.0061***
Sex[F]	0.25	-0.12, 0.61	0.1766	0.25	-0.12, 0.61	0.1788	0.12	-0.13, 0.36	0.346	0.12	-0.12, 0.37	0.3199
Maternal MUAC	-0.1	-0.26, 0.05	0.1933	-0.09	-0.25, 0.06	0.2385	-0.05	-0.16, 0.06	0.3478	-0.05	-0.16, 0.06	0.3241
Parity	0.1	-0.08, 0.27	0.2621	0.11	-0.07, 0.28	0.2283	0.15	0.04, 0.27	**0.0100***	0.16	0.04, 0.28	**0.0092***
CollectionSeason[Dry]	-0.01	-0.39, 0.37	0.9613	0.01	-0.37, 0.39	0.9587	0.07	-0.87, 1.02	0.8745	0.03	-0.9, 0.96	0.9515
BirthSeason[Dry]	-0.12	-0.61, 0.37	0.6198	-0.12	-0.62, 0.38	0.6295	-0.15	-1.05, 0.76	0.7475	-0.1	-1.01, 0.8	0.8194
EBF Status @3mo [Y]	-0.2	-0.72, 0.32	0.4312	-	-	-	-0.09	-0.43, 0.25	0.5865	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.02	-0.42, 0.39	0.938	-	-	-	-0.06	-0.33, 0.4	0.6280
SEP Score	0.01	-0.14, 0.15	0.9303	0.02	-0.12, 0.16	0.7847	-0.01	-0.11, 0.08	0.7928	-0.01	-0.1, 0.09	0.8514
SecretorStatus[Non-Secretor]	0	-0.42, 0.43	0.9869	-0.01	-0.44, 0.42	0.9601	0.2	-0.09, 0.49	0.1636	0.19	-0.1, 0.48	0.1875

"Extended" predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value *P<0.01

Undecorated HMO

None of the multiple linear regression models were significant (P>0.05) in predicting relative abundance of undecorated HMO at any time point across the first 12mo of lactation using either real time or extended predictors. Model results are detailed in **Table 3.19**.

Table 3.19. Results of multiple linear regression models examining "Real time" and "Extended" predictors of undecorated HMO relative abundance across the first 12 months of lactation

	"Real time" predictors of undecorated HMO											
Time point	3mo (MS: P=0.8288)			6mo (MS: P=0.222)			9mo (MS: P=0.2400)			12mo (MS: P=0.3367)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	48.83	-0.31, 97.97	0.0513	15.81	-21.51, 53.13	0.3956	26.86	-14.86, 68.59	0.2001	22.09	-19.95, 64.13	0.2948
Sex[F]	1.26	-3.73, 6.25	0.6022	1.15	-2.35, 4.66	0.5086	1.15	-2.68, 4.99	0.5457	0.1	-3.69, 3.89	0.9575
Maternal MUAC	-0.28	-2.34, 1.79	0.7824	0.71	-0.76, 2.18	0.3349	0.27	-1.35, 1.89	0.7347	0.38	-1.27, 2.04	0.6418
Parity	-0.77	-3.38, 1.84	0.5421	0.01	-1.58, 1.59	0.9918	0.89	-0.92, 2.69	0.3254	0.69	-1.09, 2.47	0.4366
CollectionSeason[Dry]	-0.17	-5.05, 4.71	0.942	2.87	-1.37, 7.11	0.1785	0.41	-3.55, 4.36	0.8364	-4.79	-19.23, 9.64	0.5064
BirthSeason[Dry]	0.56	-5.58, 6.69	0.851	2.31	-2.81, 7.43	0.3658	0.06	-5.09, 5.21	0.9815	5.67	-8.37, 19.72	0.4192
EBF Status @3mo [Y]	-2.9	-9.2, 3.4	0.3471	2.45	-1.92, 6.82	0.2633	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-	-	-	-	-	-	-	-	-
SEP Score	0.57	-1.06, 2.19	0.4734	0.33	-0.94, 1.61	0.6018	0.57	-0.89, 2.03	0.4329	0.88	-0.6, 2.36	0.2374
SecretorStatus[Non-Secretor]	0.89	-3.85, 5.62	0.6979	5.24	1.47, 9.02	0.0078*	5.29	0.83, 9.74	0.0213*	5.09	0.66, 9.52	0.0253*
	"Extended" predictors of undecorated HMO											
Time point	9mo_3mo (MS: P=0.2395)			9mo_6mo (MS: P=0.3367)			12mo_3mo (MS: P=0.3992)			12mo_6mo (MS: P=0.4177)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	33.52	-9.83, 76.87	0.1256	27.1	-15.56, 69.75	0.2058	24.47	-18.45, 67.39	0.2561	20.37	-22.56, 63.31	0.3433
Sex[F]	1.15	-2.68, 4.97	0.5469	1.16	-2.73, 5.05	0.5493	-0.08	-3.94, 3.77	0.9652	0.17	-3.66, 4	0.931
Maternal MUAC	0.14	-1.49, 1.77	0.8634	0.26	-1.43, 1.94	0.7594	0.34	-1.34, 2.01	0.688	0.5	-1.23, 2.23	0.563
Parity	0.76	-1.05, 2.57	0.4015	0.91	-0.97, 2.78	0.3346	0.69	-1.1, 2.48	0.4394	0.56	-1.3, 2.42	0.5462
CollectionSeason[Dry]	0.1	-3.89, 4.08	0.9607	0.38	-3.67, 4.43	0.8497	-3.86	-18.66, 10.94	0.6011	-4.82	-19.39, 9.76	0.508
BirthSeason[Dry]	0.12	-5.02, 5.26	0.9622	0.11	-5.23, 5.45	0.9673	5.14	-9.09, 19.37	0.4697	5.41	-8.8, 19.62	0.4464
EBF Status @3mo [Y]	-3.02	-8.51, 2.47	0.2717	-	-	-	-1.8	-7.1, 3.5	0.4967	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.19	-4.5, 4.11	0.9274	-	-	-	1.11	-3.08, 5.31	0.5956
SEP Score	0.38	-1.12, 1.87	0.6107	0.57	-0.91, 2.05	0.4419	0.8	-0.71, 2.31	0.2897	0.88	-0.61, 2.38	0.2393
SecretorStatus[Non-Secretor]	5.46	1.01, 9.92	0.0176*	5.26	0.71, 9.82	0.0248*	4.95	0.47, 9.43	0.0313*	5.35	0.77, 9.92	0.0231*

"Extended" predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement;

CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value *P<0.01; **P<.0001

Sia-fuc HMO

Multiple linear regression models assessing potential predictors of sia-fuc HMO class relative abundances at 3, 9, and 12 month post-partum were non-significant, as were all models examining extended effects. The model assessing potential predictors of milk collected at 6 months of lactation was significant (P=0.0441) and had a large effect size (f^2=0.52). Maternal parity significantly predicted relative abundance of sia-fuc HMO at 6 months, where higher parity (by 1 offspring) predicted higher sia-fuc abundance by 0.19% (95% CIs: 0.05, 0.32%; P=0.0105). Infant EBF status at 6 months of age was also a significant predictor of relative abundance of sia-fuc HMO at 6 months, with milk from mothers of infants still EBF at 6 months predicting lower relative abundance of sia-fuc HMO by -0.45% (95% CIs: -0.84, -0.07%; P=0.0220). Full results are detailed in **Table 3.20**.

Table 3.20**.** Results of multiple linear regression models examining "Real time" and "Extended" predictors of sia-fuc HMO relative abundance across the first 12 months of lactation

	"Real time" predictors of sia-fuc HMO											
Time Point	3mo (MS: P=0.7067)			**6mo (MS: P=0.0441*)**			9mo (MS: P=0.9647)			12mo (MS: P=0.2713)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	-0.3	-8.1, 7.51	0.9373	3.35	0.06, 6.63	**0.0459***	2.2	-0.67, 5.07	0.1287	0.66	-1.08, 2.41	0.4466
Sex[F]	-0.43	-1.22, 0.37	0.2723	-0.05	-0.36, 0.25	0.7248	-0.01	-0.27, 0.25	0.941	0.03	-0.13, 0.19	0.7234
Maternal MUAC	0.09	-0.23, 0.42	0.5558	-0.11	-0.24, 0.02	0.0815	-0.05	-0.16, 0.07	0.4038	-0.01	-0.08, 0.06	0.7632
Parity	-0.04	-0.45, 0.38	0.8599	0.19	0.05, 0.32	**0.0105***	0.01	-0.11, 0.14	0.8534	0.07	-0.01, 0.14	0.0725
CollectionSeason[Dry]	-0.11	-0.88, 0.67	0.7722	0.1	-0.27, 0.47	0.5935	-0.01	-0.28, 0.27	0.9665	0.12	-0.48, 0.72	0.6793
BirthSeason[Dry]	-0.22	-1.2, 0.75	0.6403	0.03	-0.42, 0.48	0.8953	-0.08	-0.43, 0.28	0.6581	-0.22	-0.8, 0.36	0.4511
EBF Status @3mo [Y]	0.29	-0.71, 1.29	0.5517	-	-	-	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.45	-0.84, -0.07	**0.0220***	-	-	-	-	-	-
SEP Score	-0.07	-0.33, 0.19	0.5874	0.03	-0.08, 0.14	0.561	-0.04	-0.14, 0.06	0.449	-0.02	-0.08, 0.04	0.5492
SecretorStatus[Non-Secretor]	0.36	-0.39, 1.11	0.3287	-0.16	-0.49, 0.17	0.3377	0	-0.31, 0.31	0.9945	0.01	-0.18, 0.19	0.9274
	"Extended" predictors of sia-fuc HMO											
Time Point	9mo_3mo (MS: P=0.9838)			9mo_6mo (MS: P=0.9657)			12mo_3mo (MS: P=0.3505)			12mo_6mo (MS: P=0.1591)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	2.14	-0.89, 5.18	0.1608	2.33	-0.59, 5.24	0.1145	0.74	-1.05, 2.52	0.4103	0.89	-0.83, 2.62	0.3027
Sex[F]	-0.01	-0.28, 0.26	0.9421	-0.01	-0.27, 0.26	0.9616	0.02	-0.14, 0.18	0.7811	0.02	-0.13, 0.17	0.8018
Maternal MUAC	-0.05	-0.16, 0.07	0.4275	-0.06	-0.17, 0.06	0.3375	-0.01	-0.08, 0.06	0.734	-0.03	-0.09, 0.04	0.4642
Parity	0.01	-0.11, 0.14	0.8421	0.02	-0.11, 0.15	0.7353	0.07	-0.01, 0.14	0.0752	0.08	0.01, 0.16	0.0274*
CollectionSeason[Dry]	0	-0.28, 0.28	0.9835	-0.02	-0.3, 0.26	0.8924	0.15	-0.46, 0.77	0.6217	0.13	-0.46, 0.71	0.6637
BirthSeason[Dry]	-0.08	-0.44, 0.28	0.6602	-0.05	-0.42, 0.31	0.7733	-0.24	-0.83, 0.36	0.4262	-0.18	-0.76, 0.39	0.5168
EBF Status @3mo [Y]	0.03	-0.36, 0.41	0.8855	-	-	-	-0.05	-0.28, 0.17	0.6232	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.1	-0.4, 0.19	0.4872	-	-	-	-0.15	-0.32, 0.02	0.0866
SEP Score	-0.04	-0.14, 0.07	0.4881	-0.04	-0.14, 0.06	0.435	-0.02	-0.08, 0.04	0.5087	-0.02	-0.08, 0.04	0.5264
SecretorStatus[Non-Secretor]	0	-0.31, 0.31	0.9862	-0.01	-0.33, 0.3	0.926	0	-0.18, 0.19	0.9651	-0.03	-0.21, 0.16	0.7838

"Extended" predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; CollectionSeason: Season of milk sample collection; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value *P<0.01; **P<.0001

Individual HMO structures

The 71 individual HMO structures quantified in this study are listed in **Table 3.21**. The individual HMO structures are used only in exploratory analyses in subsequent Chapters. Full details on the relative abundance of each individual structure can be found detailed in **Appendix Table A.5.**

Table 3.21. Individual HMO structures quantified in the present analysis

HMO Structure	Name
6'SL	6'-Sialyllactose
3'SL	3'-Sialyllactose
3'FL	3'-Fucosyllactose
2010a	No literature name
2010b	No literature name
2010c	No literature name
2'FL	2'-Fucosyllactose
2020a	No literature name
2020b	No literature name
LDFT	Lactodifucotetraose
2100a	No literature name
2100b	No literature name
3000a	No literature name
3000b	No literature name
3000c	No literature name
3000d	No literature name
3000e	No literature name
LNT	*Lacto-N-tetraose*
LSTc	*Sialyllacto-N-tetraose [c]*
LSTb	*Sialyllacto-N-tetraose [b]*
LNFP II	*Lacto-N-fucopentaose II*
LNFP I + III	*Lacto-N-fucopentaose I + III*
LNFP V	Lacto-N-fucopentaose V
3110b	No literature name
3110c	No literature name
3110d	No literature name
F-LSTc	*Monofucosylmonosialyllacto-N-neotetraose*
LNDFH I	Lacto-N-difucohexaose I
LNDFH II	Lacto-N-difucohexaose II
3120a	No literature name
3120b	No literature name
3200a	No literature name

HMO Structure	Name
3200b	No literature name
4100a	No literature name
4100b	No literature name
4110a	No literature name
4110b	No literature name
LNH	*Lacto-N-hexaose*
LNnH	*Lacto-N-neohexaose*
p-LNH	*para-lacto-N-hexaose*
S-LNnH II	*sialyllacto-N-neohexaose II*
MFpLNH IV	*Fucosyl-para-lacto-N-hexaose*
4120a	No literature name
MFLNH I + III	*Monofucosyllacto-N-hexaose I + III*
IFLNH III	*Isomer 3 fucosyl-para-lacto-N-hexaose*
4210a	No literature name
4210b	No literature name
IFLNH I	*Isomer 1 fucosyl-para-lacto-N-hexaose*
FS-LNnH I	Fucosylsialyllacto-N-neohexaose I
4220a	No literature name
4220b	No literature name
DFLNHa	Difucosyllacto-*N*-hexaose (a)
DFLNHb	(Difucosyllacto-*N*-hexaose (b)
DFpLNH II	*Difucosyl-para-lacto-N-hexaose*
DFS-LNnH	*Difucosylmonosialyllacto-N-neohexaose*
4230a	No literature name
TFLNH	*Trifucosyllacto-N-hexaose*
5300a	No literature name
5300b	No literature name
5301a	No literature name
5310a	No literature name
5310b	No literature name
5310c	No literature name
FS-LNO	Fucosylsialyllacto-N-octaose
DFLNO I	*Difucosyllacto-N-octaose I*
DFLNNnO II	*Difucosyllacto-N-neooactaose II*
5320a	No literature name
6400a	No literature name
6410a	No literature name
6410b	No literature name

Relative abundance of maternal milk HMGPs

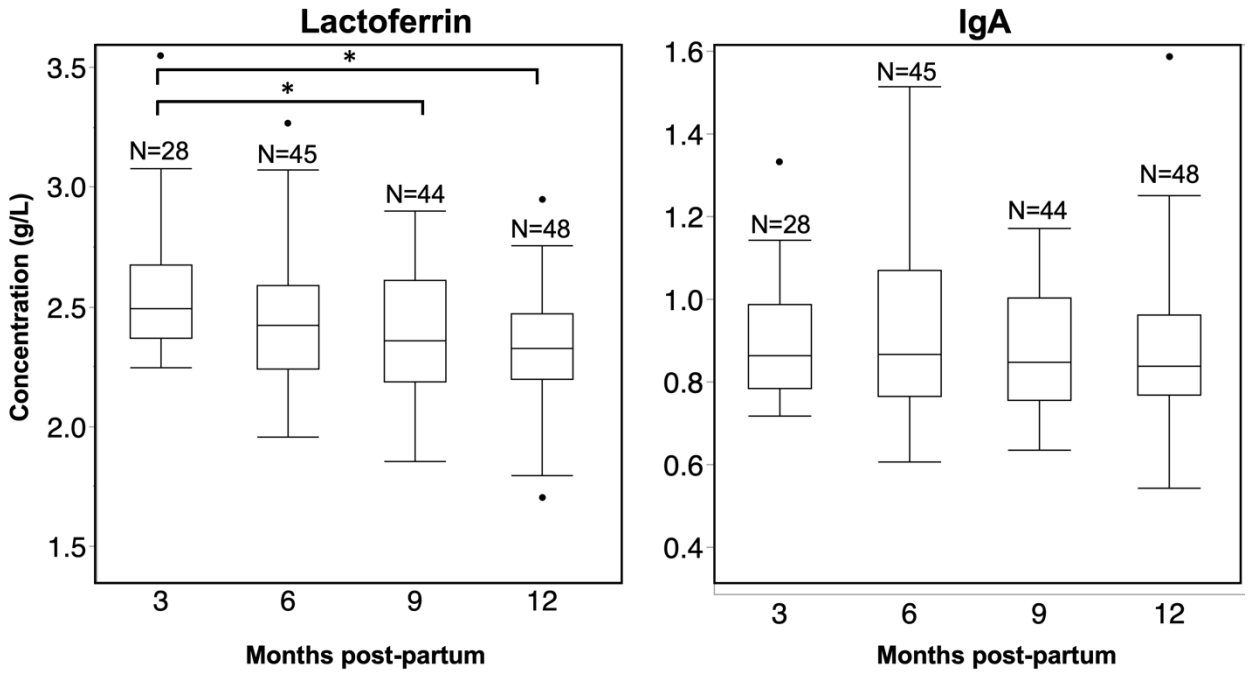
Lactoferrin

Maternal milk lactoferrin concentrations (g/L) declined across the first 12 months of lactation (**Figure 3.3).** Wilcoxon rank sums test showed that average lactoferrin concentration was significantly higher at 3 months (2.56 ±0.3 g/L) compared to 9 (2.38 ±0.3 g/L; P=0.0132) and 12 months (2.33 ±0.3 g/L; P=0.0012) of lactation, but not 6 months (2.45 ±0.3 g/L) of lactation.

IgA

Maternal milk IgA concentrations remained consistent over the 12 month period, with mean (±SD) concentrations of 0.90 g/L (±0.1), 0.91 g/LL (±0.2), 0.88 g/L (±0.1), and 0.89 g/L (±0.2) at 3, 6, 9, and 12 months of lactation, respectively (**Figure 3.3**). IgA concentrations were not significantly different between collection time points.

Figure 3.3. Milk lactoferrin and IgA concentrations at 3, 6, 9, and 12 months post-partum



*P<0.05

Mixed effects model results (HMGPs)

Lactoferrin

Mixed effects model analyses showed that collection time point was a significant predictor of relative abundance of lactoferrin during the first 12 months of lactation. Milk collected at 3 months of lactation significantly predicted higher relative abundance of lactoferrin by 0.1% (95% CIs: 0.02, 0.18%; P=0.0111). Subject ID, the random variable, was also a significant predictor of relative abundance of milk lactoferrin (B: 0.02%; 95% CIs: 0, 0.04%; P=0.0141). None of the other explanatory variables were significant predictors in the model.

IgA

The only significant predictor of relative abundance of milk IgA in the mixed effects model was the random variable, Subject ID (B: 0.01%; 95% CIs: 0, 0.02%; P=0.0176). None of the other explanatory variables were significant predictors in the model.

Full model results for analyses of potential predictors of lactoferrin and IgA are detailed in **Table 3.22.**

Table 3.22. Results from mixed effects model analyses of potential predictors of lactoferrin and IgA in maternal milk across the first year of life

		LF			IgA		
	Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Fixed Effects	Intercept	2.41	1.69, 3.12	**<.0001****	0.97	0.5, 1.45	**0.0002****
	CollectionTime point[3]	0.1	0.02, 0.18	**0.0111***	0	-0.05, 0.05	0.8843
	CollectionTime point[6]	0.03	-0.04, 0.09	0.3848	0.02	-0.02, 0.06	0.394
	CollectionTime point[9]	-0.04	-0.11, 0.02	0.1669	-0.01	-0.05, 0.03	0.571
	CollectionTime point[12] *Referent*	-	-	-	-	-	-
	Maternal MUAC	0	-0.03, 0.03	0.9229	0	-0.02, 0.01	0.7227
	SEP Score	0.02	0, 0.04	0.0925	0.01	0, 0.03	0.1726
	CollectionSeason[Dry]	0	-0.04, 0.04	0.9706	0	-0.03, 0.02	0.7897
	EBF Duration (mo)	0.01	-0.03, 0.05	0.5481	-0.01	-0.04, 0.01	0.3571
	Sex[F]	-0.03	-0.09, 0.04	0.3978	-0.01	-0.05, 0.03	0.6778
	BirthSeason[Dry]	-0.01	-0.09, 0.06	0.7022	0.01	-0.05, 0.06	0.7906
	Parity	0	-0.03, 0.03	0.8321	0.01	-0.01, 0.3	0.1635
	SecretorStatus[Non-Secretor]	0.04	-0.03, 0.1	0.2661	0.01	-0.03, 0.05	0.5909
Random Effect	SubjectID	0.02	0, 0.04	**0.0141***	0.01	0, 0.02	**0.0176***

B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value; *P<.05; **P<0.01 Collection season: Season of milk collection (Dry vs Wet)

Multiple linear regression model results (HMGPs)

Milk lactoferrin

None of the multiple linear regression models were significant (P>0.05) in predicting relative abundance of milk lactoferrin at any time point across the first 12 months of lactation using either real time or extended predictors. Results from the statistical models are detailed in **Table 3.23.**

Table 3.23. Results of multiple linear regression models examining “Real time” and “Extended” predictors of milk lactoferrin across the first 12 months of lactation

	"Real time" predictors of lactoferrin											
Time Point	3mo (MS: P=0.9545)			6mo (MS: P=0.9908)			9mo (MS: P=0.6975)			12mo (MS: P=0.1403)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	2.93	1.34, 4.51	0.0011*	2.37	1.19, 3.56	0.0003*	1.96	0.96, 2.97	0.0004*	2.87	2.02, 3.73	<.0001*
Sex[F]	-0.04	-0.2, 0.12	0.6274	-0.04	-0.15, 0.07	0.4958	-0.03	-0.12, 0.06	0.5407	-0.01	-0.09, 0.07	0.7651
Maternal MUAC	-0.02	-0.08, 0.05	0.5739	0	-0.05, 0.05	0.9849	0.02	-0.02, 0.06	0.3141	-0.02	-0.06, 0.01	0.1922
Parity	0.01	-0.08, 0.09	0.8436	0	-0.05, 0.05	0.9896	-0.01	-0.06, 0.03	0.5012	0.01	-0.03, 0.04	0.6868
CollectionSeason[Dry]	-0.02	-0.18, 0.13	0.7708	0.01	-0.13, 0.14	0.9035	0	-0.1, 0.09	0.947	-0.16	-0.45, 0.13	0.264
BirthSeason[Dry]	0.02	-0.18, 0.21	0.863	0.04	-0.13, 0.2	0.6505	-0.09	-0.22, 0.03	0.1375	0.15	-0.13, 0.43	0.2956
EBF Status @3mo [Y]	0.05	-0.15, 0.25	0.6258	-	-	-	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.06	-0.2, 0.08	0.3876	-	-	-	-	-	-
SEP Score	0.02	-0.03, 0.07	0.3908	0.01	-0.03, 0.05	0.7013	0.01	-0.02, 0.05	0.445	0.04	0.01, 0.07	0.0150*
SecretorStatus[Non-Secretor]	0.01	-0.14, 0.16	0.8731	-0.01	-0.13, 0.11	0.8102	0.02	-0.08, 0.13	0.6663	0.05	-0.04, 0.14	0.2333
	"Extended" predictors of lactoferrin											
Time Point	9mo_3mo (MS: P=0.7963)			9mo_6mo (MS: P=0.6351)			12mo_3mo (MS: P=0.1403)			12mo_6mo (MS: P=0.1853)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	1.97	0.91, 3.03	0.0006*	1.86	0.84, 2.88	0.0007*	2.76	1.92, 3.6	<.0001*	2.95	2.1, 3.79	<.0001*
Sex[F]	-0.03	-0.12, 0.07	0.5458	-0.03	-0.12, 0.06	0.4745	0	-0.08, 0.07	0.9495	-0.02	-0.09, 0.06	0.6885
Maternal MUAC	0.02	-0.02, 0.06	0.3279	0.03	-0.01, 0.07	0.1989	-0.02	-0.05, 0.01	0.236	-0.03	-0.06, 0.01	0.1117
ChildrenPrior	-0.01	-0.06, 0.03	0.5069	-0.02	-0.07, 0.02	0.3311	0.01	-0.03, 0.04	0.6833	0.01	-0.02, 0.05	0.4526
CollectionSeason[Dry]	0	-0.1, 0.09	0.9415	0	-0.09, 0.1	0.9675	-0.21	-0.5, 0.08	0.1509	-0.16	-0.45, 0.13	0.2643
BirthSeason[Dry]	-0.09	-0.22, 0.03	0.1433	-0.11	-0.24, 0.02	0.0845	0.17	-0.1, 0.45	0.2103	0.16	-0.12, 0.44	0.2513
EBF Status @3mo [Y]	0	-0.14, 0.13	0.9564	-	-	-	0.09	-0.01, 0.19	0.088	-	-	-
EBF Status @6mo[Y]	-	-	-	0.06	-0.04, 0.16	0.2459	-	-	-	-0.06	-0.14, 0.02	0.1537
SEP Score	0.01	-0.02, 0.05	0.4715	0.01	-0.02, 0.05	0.4092	0.04	0.01, 0.07	0.0071*	0.04	0.01, 0.07	0.0147*
SecretorStatus[Non-Secretor]	0.02	-0.08, 0.13	0.6686	0.03	-0.07, 0.14	0.544	0.06	-0.03, 0.15	0.1694	0.04	-0.05, 0.13	0.3722

“Extended” predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound)

Milk IgA

None of the multiple linear regression models were significant (P>0.05) in predicting relative abundance of milk IgA at any time point across the first 12 months of lactation using either real time or extended predictors. Results from the statistical models are detailed in **Table 3.24**.

Table 3.24. Results of multiple linear regression models examining “Real time” and “Extended” predictors of milk IgA across the first 12 months of lactation

	"Real time" predictors of IgA											
Time Point	3mo (MS: P=0.8836)			6mo (MS: P=0.5764)			9mo (MS: P=0.807)			12mo (MS: P=0.5201)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	0.78	-0.01, 1.56	0.0514	0.68	-0.06, 1.43	0.0714	0.97	0.38, 1.56	0.0019*	1.01	0.33, 1.69	0.0046*
Sex[F]	-0.04	-0.12, 0.04	0.2893	-0.05	-0.12, 0.02	0.1919	0.01	-0.04, 0.07	0.6601	0.02	-0.04, 0.09	0.4518
Maternal MUAC	0	-0.03, 0.04	0.7713	0	-0.03, 0.03	0.8889	0	-0.03, 0.02	0.8266	-0.01	-0.04, 0.02	0.472
Parity	-0.01	-0.05, 0.03	0.6077	0.02	-0.01, 0.05	0.1443	0	-0.03, 0.02	0.9611	0.03	0, 0.06	0.0419*
CollectionSeason[Dry]	0	-0.08, 0.08	0.9759	0.02	-0.06, 0.11	0.5704	0	-0.06, 0.06	0.9972	-0.07	-0.3, 0.16	0.5298
BirthSeason[Dry]	0.01	-0.09, 0.1	0.9097	0.06	-0.04, 0.17	0.2105	-0.05	-0.12, 0.02	0.1762	0.07	-0.16, 0.29	0.557
EBF Status @3mo [Y]	0	-0.1, 0.1	0.9477	-	-	-	-	-	-	-	-	-
EBF Status @6mo[Y]	-	-	-	-0.05	-0.14, 0.04	0.2403	-	-	-	-	-	-
SEP Score	0.02	-0.01, 0.04	0.142	0.02	-0.01, 0.04	0.1953	0.01	-0.01, 0.03	0.5704	0.01	-0.02, 0.03	0.5647
SecretorStatus[Non-Secretor]	0	-0.08, 0.07	0.9142	-0.02	-0.09, 0.06	0.6358	0.01	-0.06, 0.07	0.8256	-0.02	-0.09, 0.05	0.5637
	"Extended" predictors of IgA											
Time Point	9mo_3mo (MS: P=0.8053)			9mo_6mo (MS: P=0.7984)			12mo_3mo (MS: P=0.5590)			12mo_6mo (MS: P=0.4216)		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	1.04	0.43, 1.66	0.0015*	0.93	0.33, 1.53	0.0035*	1.05	0.36, 1.74	0.0038*	1.06	0.39, 1.74	0.0029*
Sex[F]	0.01	-0.04, 0.07	0.6785	0.01	-0.04, 0.06	0.7213	0.02	-0.04, 0.08	0.5278	0.02	-0.04, 0.08	0.5055
Maternal MUAC	0	-0.03, 0.02	0.7468	0	-0.02, 0.02	0.9696	-0.01	-0.04, 0.02	0.4338	-0.01	-0.04, 0.01	0.3151
Parity	-0.03	-0.11, 0.04	0.3841	0	-0.03, 0.02	0.7635	0.03	0, 0.06	0.0427*	0.03	0.01, 0.06	0.0211*
CollectionSeason[Dry]	0	-0.03, 0.02	0.8605	0	-0.05, 0.06	0.9363	-0.06	-0.29, 0.18	0.6406	-0.07	-0.3, 0.16	0.5352
BirthSeason[Dry]	0	-0.06, 0.05	0.886	-0.06	-0.13, 0.02	0.1269	0.06	-0.17, 0.28	0.6222	0.08	-0.15, 0.3	0.4969
EBF Status @3mo [Y]	-0.05	-0.12, 0.02	0.1777	-	-	-	-0.03	-0.12, 0.05	0.4221	-	-	-
EBF Status @6mo[Y]	-	-	-	0.03	-0.03, 0.09	0.3652	-	-	-	-0.05	-0.11, 0.02	0.1721
SEP Score	0	-0.02, 0.02	0.7269	0.01	-0.01, 0.03	0.5411	0.01	-0.02, 0.03	0.6565	0.01	-0.02, 0.03	0.5766
SecretorStatus[Non-Secretor]	0.01	-0.05, 0.07	0.7697	0.01	-0.05, 0.07	0.7215	-0.02	-0.1, 0.05	0.5175	-0.03	-0.1, 0.04	0.3939

“Extended” predictor time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary explanatory variables of interest (e.g., 9mo_3mo = 9mo milk composition in relationship to 3mo EBF status); Maternal MUAC: 3rd trimester maternal MUAC measurement; MS: Model significance; B: Coefficient estimate; LB, UB: 95% CIs (Lower Bound, Upper Bound); P: P-value

Associations between milk constituents

Correlations between the individual milk constituents measured here were visually inspected in a correlation matrix. Displayed in **Table 3.25** through **Table 3.28.** the relationships between maternal milk constituents are shown from 3, 6, 9, and 12 months post-partum. This investigation showed collinearity between multiple nutritional and bioactive milk components between the four time points. The strongest correlation was between fucosylated and undecorated HMO structures (R^2=-0.9830), followed by lactoferrin and IgA (R^2=0.9086), protein and TRP (R^2=0.8452), sialylated and sia-fuc HMOs (R^2=0.7601), and protein and sialylated HMO (R^2=0.7192). The full results from the correlation analyses are presented as R^2 values and are detailed in Table 3.25.

Table 3.25. Correlation matrix of associations between maternal milk macronutrient, HMO, and HMGP composition (3 months post-partum)

Fat	**Fat**									
Protein	-0.0342	**Protein**								
Lactose	-0.4829	-0.3657	**Lactose**							
TRP	0.2708	**0.8152**	-0.3614	**TRP**						
Fuc	-0.1771	-0.0658	0.1438	-0.1077	**Fuc**					
Sia	0.0310	0.4469	-0.1059	0.3894	-0.3938	**Sia**				
Undec	0.1726	-0.0638	-0.1321	0.0242	**-0.9781**	0.2168	**Undec**			
Sia-fuc	-0.0309	0.4298	0.0741	0.1222	0.5245	-0.0114	-0.6336	**Sia-fuc**		
LF	-0.0863	0.5327	-0.3541	0.0792	-0.0591	0.2847	-0.0332	0.3532	**LF**	
IgA	-0.1733	0.4863	-0.2435	0.0544	-0.0523	0.3629	-0.0435	0.2710	**0.8972**	**IgA**

R^2>0.8 indicated by cell border and boldface font

Table 3.26. Correlation matrix of associations between maternal milk macronutrient, HMO, and HMGP composition (6 months post-partum)

Fat	**Fat**									
Protein	0.1515	**Protein**								
Lactose	-0.3805	-0.3919	**Lactose**							
TRP	0.3078	**0.8653**	-0.5015	**TRP**						
Fuc	0.2029	-0.0488	-0.2259	-0.0814	**Fuc**					
Sia	-0.0197	0.3678	-0.3795	0.4744	-0.3217	**Sia**				
Undec	-0.2128	-0.0343	0.3271	-0.0265	**-0.9822**	0.1518	**Undec**			
Sia-fuc	0.1241	0.4018	-0.5729	0.5281	0.1968	0.5124	-0.3545	**Sia-fuc**		
LF	0.1104	0.2998	-0.4595	0.2774	0.1707	0.3387	-0.2618	0.5207	**LF**	
IgA	0.1549	0.3339	-0.6290	0.3803	0.3959	0.2586	-0.4836	0.5808	0.5893	**IgA**

$R^2 > 0.8$ indicated by cell border and boldface font.

Table 3.27. Correlation matrix of associations between maternal milk macronutrient, HMO, and HMGP composition (9 months post-partum)

Fat	**Fat**									
Protein	-0.1195	**Protein**								
Lactose	-0.3444	-0.3108	**Lactose**							
TRP	-0.1212	0.4169	-0.1376	**TRP**						
Fuc	0.0940	-0.2944	0.0458	-0.0801	**Fuc**					
Sia	-0.0977	0.4204	-0.5323	0.1970	-0.4853	**Sia**				
Undec	-0.0947	0.2514	0.0171	0.0618	**-0.9944**	0.3951	**Undec**			
Sia-fuc	0.1233	0.3723	-0.4721	0.1043	0.1355	0.5178	-0.2272	**Sia-fuc**		
LF	0.0075	0.1724	-0.4499	0.2792	0.2041	0.2516	-0.2545	0.5337	**LF**	
IgA	-0.1576	0.1200	-0.3115	0.2847	0.2514	0.2043	-0.2933	0.4037	**0.9398**	**IgA**

$R^2 > 0.8$ indicated by cell border and boldface font.

Table 3.28. Correlation matrix of associations between maternal milk macronutrient, HMO, and HMGP composition (12 months post-partum)

Fat	**Fat**									
Protein	0.3498	**Protein**								
Lactose	-0.7332	-0.5102	**Lactose**							
TRP	0.3818	**0.8392**	-0.3351	**TRP**						
Fuc	-0.3418	-0.4132	0.3352	-0.4122	**Fuc**					
Sia	0.3302	0.4237	-0.4341	0.3823	-0.4480	**Sia**				
Undec	0.3258	0.3873	-0.3086	0.3917	**-0.9964**	0.3734	**Undec**			
Sia-fuc	0.0446	0.2123	-0.1580	0.1273	0.1213	0.5516	-0.1925	**Sia-fuc**		
LF	0.0418	-0.0040	-0.0899	-0.1061	0.0877	0.3850	-0.1268	0.3734	**LF**	
IgA	-0.0335	-0.0156	-0.0677	-0.0792	0.1127	0.2430	-0.1398	0.2738	**0.9136**	**IgA**

R^2>0.8 indicated by cell border and boldface font

DISCUSSION

Millions of years of evolution have shaped the complex, dynamic, and highly variable composition of human milk to meet nutritional and immunological needs of the developing infant. Maternal milk composition between individuals and across the course of lactation, allowing for unique milk profiles that can meet infant immunological and nutritional needs. Investigations between maternal, infant, and environmental factors and maternal milk composition in a subset of participants from the HERO-G project are discussed below.

Mixed effects model analyses showed that milk collection time point was a significant predictor of milk composition for all constituents in this analysis except lactose and milk IgA. This indicates that lactation stage is an important driver of milk composition during the first 12 months of lactation, which has been previously established in the literature. Furthermore, this finding provides justification for constructing multiple linear models separated by time point, which allow for more detailed assessment of temporally influenced shifts in maternal milk profiles. Because the introduction of non-breast milk foods (~5mo of age in this population) is a particularly immunologically challenging period for offspring, individual time point assessments may show compositional shifts in maternal milk that mirror changes in offspring needs as it relates to dietary changes. The absence of influence of collection time point on lactose and milk IgA is discussed in later paragraphs.

Besides collection time point and Subject ID, none of the explanatory variables were significant predictors of any of the maternal milk constituents except fat and TRP. Milk fat was significantly predicted by infant sex, with female offspring predicting higher milk fat relative to milk from mothers of male offspring. Studies of baboon milk composition report higher energy and fat content for male versus female offspring[28]. Research in Korean and Kenyan mothers found that maternal milk was higher in energy for female offspring than males[441]. The mechanisms driving sex-specific differences in milk profiles are not yet fully elucidated and require further investigations. A more detailed understanding of sex-specific differences in milk composition will improve our understanding of unequal parental investment, which may also be indicative of maternal condition.

Of particular interest in this dissertation is the impact of seasonality in The Gambia on infant feeding, including maternal milk composition. Here, the only milk constituent with evidence of being influenced by seasonality was TRP. The mixed effects model and the multiple linear regression model at 6mo both showed that TRP was sensitive to seasonality, with milk collected during the dry (“harvest”) season predicting greater concentrations of TRP relative to milk collected during the wet (“hungry”) season. Bioactive factors in milk have been shown to be

seasonally influenced in other populations[442]. TRP in maternal milk is sourced from maternal circulation, thus the concentration in maternal milk may reflect a mother's own health or immune challenges. Infant needs may also be the driver of milk profiles. In The Gambia, the annual rains create an environment more conducive for infection and illness, and they influence food availability as the majority of staple crops are in stages of growth, not ready for harvest, during the wet season. These pressures associated with seasonality create a context through which having greater TRP content in milk may be related to needs and energy availability of both mother and infant. Older studies from The Gambia report that little agricultural work is conducted between January to March – months which fall during the dry season – and reduced agricultural workloads for mothers during this period may in turn result in greater energetic resource availability for production of certain milk constituents, particularly those which provide offspring with immunological support[374].

It is important to note that 6 months was the only time point in which TRP was predicted by seasonality. This coincides with the period in which infants are particularly immunologically vulnerable – when non-breast milk foods are being introduced and when they are coming into contact with foreign substances and microbes – and when they are experiencing rapid growth. TRP has been shown to influence production of growth factors that are important in early life somatic growth and aid in development of infant immune system defenses[443,444]. Here, TRP concentrations match averages seen in other populations, though data on TRP concentrations after 6 months of age are limited[175,354,445–449]. A significant reduction in milk protein and true protein across the first year of lactation may reflect a reduction of maternal investment in offspring as she transitions towards her own self-maintenance or investment in other reproductive events, along with supplemented infant diet by non-breast milk foods. In humans, milk protein concentration is not impacted by maternal diet; however, it has been shown to increase with maternal weight-for-height and decrease in mothers producing higher volumes of milk[31].

While there was a significant impact of seasonality on milk TRP, lactoferrin and IgA were not predicted by season of milk production across the first 12 months of lactation. Lactoferrin and immunoglobulins are among the principal proteins in human milk, and play important roles beyond nutritional support. Growing evidence suggests that production of milk-specific immune factors is energetically costly. As such, they are a form of maternal investment in offspring outcomes that is subject to life history tradeoffs.

HMGPs were not predicted by any of the explanatory variables included in the multiple linear regression or mixed effects models. Some previous studies have found evidence to suggest that parity, nutritional status and energy

expenditure are connected to the production of milk IgA, though others have not found the same link[436]. Miller & McConnell (2014) report that the lack of relationship between milk IgA and maternal nutritional status in their study among Ariaal women in Northern Kenya may relate to the chronic undernutrition prevalent in the population. Other work conducted in the area showed that severe undernutrition masked evidence of reproductive-related changes in nutritional status[450].

Reduction in relative abundance of milk constituents over the lactation period may reflect a shift in maternal investment from current offspring to either future offspring or to maternal self-maintenance. The consistent production of certain milk components may indicate their continued value to offspring. For example, the lack of variation in the abundance of maternal milk IgA across the first 12 months of lactation seen in this analysis may suggest resilient investment in offspring despite other physiological and environmental challenges a mother may be facing at the time. Studies in other populations show steep declines in milk IgA during the early months of lactation, particularly between 3 and 6 months of age. This unique pattern – the lack of sharp decline in IgA content in Gambian maternal milk – was noted in Miller & McConnell (2015) in a population comparison of milk IgA. Chronic inflammation or selective environmental pressures on the maternal immune system may be a source of this pattern in this population. In previous studies in The Gambia, milk IgA production was sensitive to seasonality, with mothers producing less IgA in their milk during the wet season when food stores are depleted and more IgA during the dry season[386]. However, there was no difference in milk IgA based on seasonality in the present analysis. Differences between the present analysis and previous work in The Gambia may be attributable to differences in analytical technique. Further investigations into a greater variety of milk immunoglobulins in relationship to seasonality may be informative in the interpretation of the difference in these results.

Little is known about the relationships between maternal nutrition during pregnancy and milk composition throughout lactation. Here, there was no evidence to support an influence of maternal nutritional status (as defined by maternal MUAC measurements) during the third trimester on milk composition. For the purposes of this dissertation, the use of third trimester maternal MUAC measurements serves as a general proxy of maternal condition/nutritional status near the start of lactation. Collecting and assessing anthropometric measurements of maternal nutritional status (such as MUAC) in relationship to milk composition during the course of lactation is the most common approach used in the literature; however, there is some evidence in the existing literature that demonstrates that second and third trimester MUAC measurements are adequate markers of maternal malnutrition (including both under- and

overnutrition)[266,267]. Given the available data from the HERO-G study and existing literature on the topic, maternal MUAC during the third trimester is an appropriate time point to use as a marker of nutritional status. Another option that was considered here was using delta MUAC, however, there was a strong correlation in MUAC measurements across pregnancy and no clear outliers on either extreme. This approach would perhaps be better suited for studies focusing on populations with severe under or overnutrition and/or in the presence of significant increase or decrease in MUAC across time points. For the purposes of this study, using MUAC at a single measurement is considered suitable. Finally, the third trimester is also a time period which has been shown to be a strong predictor of infant birth outcomes and a window in which maternal nutritional supplements have proven influential on infant birth weight[236,451]. This is relevant to subsequent Chapters investigating infant growth outcomes.

The number of children a mother has breastfed can be used as a proxy for the number of lactation cycles she has experienced. The current analysis did not find any effects of parity on milk macronutrient or HMGP composition. Without any clear relationship between macronutrients and HMGPs and maternal parity, evidence does not support a shift in investment in current offspring towards future offspring as measured by these specific milk constituents. However, a relationship was found between parity and relative abundance of sialylated and sia-fuc HMO classes, which may relate to hormonally regulated processes of glycogenesis, though this area of research is still growing[452].

Lactose concentrations were not significantly associated with any of the explanatory variables included in the models. As the principle sugar of human milk, lactose accounts for ~40% of the energy content in milk[453]. It is the least variable milk macronutrient. Because HMOs share a common backbone of the sugar lactose, the concentration of this primary milk sugar is also broadly representative of the HMO classes and structures. Future studies examining the finer contributions of individual HMO structures (71 individual structures were quantified in the present analysis) will help bridge knowledge gaps between our broader understanding of the role of lactose relative to the nuanced contributions of unique HMO structures.

There was unexpected variation in phenotypic Secretor status over the course of lactation in four of the mothers in this analysis. Specifically, the designated Secretor status varied at one time point across the first 12 months of lactation, whereas genotypically we would expect to see uniform designation across the lactation period. Others have reported similar outcomes[421] where a mother's phenotypic Secretor status shifts over time when determined by milk composition alone as opposed to blood group (genotype obtained by PCR). This may illustrate the degree of individual plasticity in milk profiles over time. Additional research is necessary to understand evolutionary

implications of this potential flexibility in milk phenotype and the potential mechanisms driving this variation. One consideration for future analyses may be to incorporate measures of infant Secretor status in addition to maternal status. In a study of vaccine performance in Bangladesh, maternal Secretor status significantly affected vaccine immunogenicity[454]. Though infant Secretor status was not a significant predictor of seroconversion, the effect of maternal status on vaccine performance was more pronounced in Secretor infants than non-Secretor infants. Shifts in maternal HMO profiles may be dependent, in part, upon infant immunological needs, which may be determined by infant's own Secretor status. Similarly, Subject ID, used in the mixed effects models to control for intraindividual variation, was a significant predictor of all of the milk constituents quantified in this analysis. This finding highlights the natural biological variation between mother-infant dyads, which may not be adequately summarized by population level studies.

Assessments of correlation showed several strong connections between concentrations/abundance of nutritional and bioactive components of maternal milk in the analysis subset. The strongest correlations were between fucosylated and undecorated HMO structures, followed by protein and TRP, and lactoferrin and IgA. Structural similarities between milk components likely contributes to the correlations. Certain functional aspects or mechanistic elements (which can also relate to structure – particularly in HMOs) of these particular milk components involve coordination with one another or may change in concert with one another during, for example, an immunological response to infection. Another possibility is that maternal production of certain milk constituents may be more energetically costly than others, which may drive certain nutritional and bioactive milk profiles. The finding of strong collinearity will be incorporated in subsequent Chapters when maternal milk composition is examined via statistical models in relationship to infant health and growth outcomes.

There were limitations to this study. First, milk volumes consumed by infants were not recorded as a part of the HERO-G project; differences in the volume of milk production/consumption may influence effects on macronutrient concentrations (if they are volume-dependent)[338]. Evaluating true caloric intake and thereby how variation in milk macronutrients (or other bioactive molecules) may affect infant growth and health should be interpreted with this context in mind. In The Gambia, prior studies have shown that 12-hour maternal milk output volume steadily declines during the wet season, with milk output being lowest in August through October[401,455–457]. As such, there is a tendency for rural Gambian infants to have a reduced intake of maternal milk as subsistence activities increase[225,299,458,459]. The time between birth and 24 months of age has been described as a window of

opportunity to address undernutrition[159,460]. Thus, the available data here are still reflective of nutritional intake and shifts in diet during a critical period of time during human development. Additionally, evidence suggests that that milk IgA does not vary based on milk volume.

Inclusion of relative abundances of individual HMO structures was beyond the scope of the analyses conducted in this dissertation. There is great value in a more detailed assessment of the variation in HMO structures within a population and over the course of lactation. Mounting evidence suggests that maternal synthesis of HMOs varies widely, yet we still do not fully understand the level of variation or the finer details of the underlying mechanisms. Future investigation showing the extent to which milk composition responds to specific environmental pressures may help us to understand the great interindividual variation noted in this study. In particular, examining individual HMO structures will be a useful supplemental analysis.

A notable strength of this study is the longitudinal design of sample collection. This allows for a clearer assessment of shifts in milk composition over time and the potential drivers of these patterns in the target population at both the group and individual level. It also creates the ability to identify and relate events to particular exposures or explanatory variables, and to further characterize the exposures with regard to presence, timing, and chronicity. Another strength of this study is that all milk samples included in the HMO and HMGP analysis were analyzed at the same time in the same laboratory. Additionally, all samples selected for this analysis were from the right breast, allowing for additional consistency and reduced source of potential variation. It is also Muslim tradition that all feeds should begin with the right breast (88% of all observed feeds in one rural Gambian study started on the right breast[261]), though there is mixed evidence regarding significant between-breast differences in milk composition.

CONCLUSION

The goal of Chapter 3 was to characterize maternal milk nutrition and bioactive composition across the first year of lactation in a subset of individuals from the larger HERO-G cohort. Concentrations of macronutrients (fat, protein, lactose, and true protein) and relative abundances of human milk oligosaccharides (fucosylated, sialylated, undectorated, and sia-fuc HMO structures) and human milk glycoproteins (lactoferrin and IgA) were assessed. Milk composition aligned with results from other studies quantifying macronutrients and previous studies in this region quantifying HMOs and human milk glycoproteins. The statistical analyses showed numerous influential factors on maternal milk composition during the first 12 months of lactation. Seasonality was an important driver of maternal

milk TRP and protein content in PLS models, which may reflect seasonal pressures on maternal and/or infant immune function, as TRP and protein are macronutrients which encompass many individual immune proteins. A more detailed investigation of maternal agricultural workload (including details on specific agricultural task types, duration of time away from infant while working, etc.), and maternal diet/nutritional status at the time of milk production, could provide more context for interpreting these findings. Collection time point, representative of stage of lactation, also significantly predicted maternal milk composition. This trend has been commonly noted across virtually all human populations. It may relate to temporally-driven pressures on milk constituents and shifts in infant immunological/nutritional needs as complementary foods are being introduced. It may also relate to maternal energetic investment in offspring, which can shift depending on tradeoffs between current and future offspring requirements.

Evaluating drivers of variation in maternal milk composition is critical in understanding how shifts in environment or physiology may contribute to the composition of early life dietary intake and subsequent infant health and growth outcomes. Physiological and nutritional cues received during early life can significantly affect metabolic and immune defense functionality in the short- and long-term. Morbidity events such as infections, chronic inflammation, and intestinal permeability can impact the infant's ability to utilize nutrients from their dietary intake, which can subsequently impact weight and height outcomes. Suboptimal diet in particular during early life can contribute to impaired innate and acquired mucosal defenses, which can ultimately increase an individual's susceptibility to later-life morbidities. As explored in Chapter 2, early introduction of non-breast milk foods and early cessation of exclusive breastfeeding has been associated with increased intestinal permeability, an overactive immune system response. Maternal milk conveys valuable nutritional and immunological information to the infant. In Chapter 4, I will assess the relationships between maternal milk composition and infant morbidity occurrence.

CHAPTER 4. ANALYSIS OF MORBIDITY OCCURRENCE

INTRODUCTION

Maternal milk provides passive immunity to infants during early life, and ample research supports that longer exclusive breastfeeding duration positively impacts offspring health and development[217,461,462]. While the immature infant immune system develops, maternal milk provides passive immunity to the infant, transferring specific components capable of defending against infections common to the local environment and to pathogens the mother was exposed to over her own lifetime[188,216,365]. The established benefits of breastfeeding are particularly critical in low-income counties, where diarrheal disease and other morbidities are common and significantly increase risk of mortality in children under 5 years of age[87,357–361]. These findings contributed to the World Health Organization (WHO) global recommendation of exclusive breastfeeding infants to 6 months of age[273].

The weaning period can be particularly dangerous for infants due to exposure to novel foods, environments, and thus pathogens and contamination. Nutrition and infection during early life have complex cyclical interactions with lasting impacts on health and development[361,463,464]. Whereas early weaning can trigger chronic inflammatory responses which can lead to infant morbidities, longer exclusive breastfeeding duration is linked to a significant reduction in the prevalence of diarrheal-related diseases and respiratory infections[465–467]. The introduction of non-breast milk foods requires caution because of the associated increased risk of exposure to contaminated foods or water containing foreign pathogens, which can greatly challenge the immature infant immune system[361,468–470].

Rates of under 5 mortality from diarrhea and pneumonia in The Gambia are high and peak during complementary feeding age[467]. Breastfeeding is a nearly universal practice in The Gambia, however, the nuanced effects of breastfeeding duration, the types and timing of introduction of non-breast milk foods, together with other individual maternal and infant characteristics, can result in variation in infant health outcomes. Evaluating the possible impacts of human milk intake, exclusive breastfeeding duration, and introduction of non-breast milk foods on infant morbidity provides a framework to examine offspring health outcomes as a result of early life nutritional environments.

In this Chapter, I present an overview of research on infant morbidity in The Gambia in relationship to early life diet. Then I provide a descriptive analysis of infant morbidities in the first year of life in the HERO-G subsample, and assess the potential influence of maternal milk composition and exclusive breastfeeding duration on infant morbidity occurrence at 3, 6, 9, and 12 months of age. To investigate possible extended effects of early life diet on

health outcomes, cumulative morbidity at 9 and 12 months of age was also assessed in relationship to exclusive breastfeeding status and maternal milk composition at 3 and 6 months of lactation.

BACKGROUND

Infant morbidity

Diarrheal disease is the second leading cause of death and a leading cause of undernutrition in children under 5, most of whom are from low-income countries and most of those deaths occurring in rural areas[471–473]. In The Gambia, a 2012 study reports that approximately 9% of deaths in children under 5 are caused by diarrheal-diseases[474]. The most recent global estimates report that sub-Saharan Africa has the highest morality rate for children under 5 years of age of any world region, with the majority of these deaths caused by preventable or treatable morbidities, such as malaria, diarrhea, and pneumonia[471,475]. Diarrhea resultant from intestinal parasites is prevalent in rural Gambia, especially between 6-23 months of age, becoming more prevalent after exclusive breastfeeding cessation[476].

Bacterial overgrowth in the small intestine is associated with growth faltering, and has been reported in high rates in children from low-income populations around the world[477,478]. Environmental enteropathy (EE), a condition that leads to chronic inflammation in the gastrointestinal tract[477,478], is characterized pathologically by villous blunting, which likely reduces the surface area of the mature absorptive intestinal epithelial cells[78]. This damage can increase intestinal permeability, allowing large macromolecules translocate into circulation, and can also reduce the concentrations of important digestive enzymes. Despite these hallmark issues associated with EE, individuals with this condition are often seemingly asymptomatic, rarely presenting overt intestinal symptoms[479].

Intestinal damage documented in a study of a rural Gambian population began around 6 months of age, coinciding with the timing of introduction of home-prepared complementary foods. Jejunal biopsies performed on Gambian children revealed a variety of pathologies; some had normal intestinal architecture while others had flattened villi, and most specimens had crypt hypertrophy and high concentrations of lymphocytes[479]. In The Gambia, previous work has identified persistent and chronic damage to the gut, increasing intestinal permeability, which is associated with poor growth outcomes[61,69,478,480]. Specifically, up to 64% of observed height and weight faltering of rural Gambian infants could be explained by increased intestinal permeability via impaired small intestinal mucosal function[69,481]. This evidence, suggestive of a pro-inflammatory state, may reflect an advantage to remaining in a hyperimmune state: constant or frequent repeated infiltration of pathogens occurring in the gut may require structural and immunological

changes in the intestines in order to survive under the severe physiological pressures of this local environment. The benefits of this state of chronic immune activation may outweigh the costs.

In addition to gastrointestinal diseases, respiratory tract infections also significantly contribute to infant weight faltering[482]. The most common infections in rural Gambian infants between birth and 6 months of age are respiratory infections (prevalence: 30.8%). Rowland et al. (1988) report that lower respiratory tract infections (LRTI) had a greater negative impact per day on weight gain than diarrhea in Gambian children. LRTI prevalence had seasonal variation in this region, with greater impacts on weight gain from August to October. Recent studies show that Gambian newborns are rapidly colonized by pneumonia-causing bacteria during the first month of life[483–485].

Relative to non-breastfed and those exclusively breastfed for less than the WHO recommended 6 months of age[321], infants who are exclusively breastfed to or beyond 6 months of age have lower risk of morbidity and mortality, particularly those related to gastrointestinal infections, which are commonplace in the rural, sub-tropical Gambian setting. Evidence shows that Gambian infants experience extreme disease burden and suffer from high rates of morbidity, with the severity particularly apparent during the wet season, when infant mortality rates increase by 10-fold[216,486–490]. Those born in the wet season are at much greater earlier risk of mortality due to infection than those born in the dry season, with these effects noted beginning in late adolescence and early adulthood[216,280,490–493]. Research has even described the region's environmental conditions as placing the "experiment of nature" on The Gambia's population[126].

Incidences of infections, including malaria, pneumonia, and diarrhea, increase during the rainy season, and birth during the wet season is correlated with higher risk of earlier mortality from infectious diseases during young adulthood in this region[216,280,490–493]. These findings have been corroborated elsewhere in West Africa[189], and authors suggest that rainfall has a negative impact on survival through the reduction in breastfeeding or premature weaning during times of increased agricultural workload. An immunological insult during early life, linked to birth season, was hypothesized to disrupt immune responses to certain infectious diseases later in life in the West Kiang region. A study in this region found significant seasonal variation of markers of immune response (T cells, B cells, mucosal barrier mechanisms and secretions), though no consistent link between birth season and immune function in rural Gambian children (aged 6.5-9.5 years of age) was detected[494]. Authors suggest, however, that individuals born during or shortly after the wet season may be impaired during early life, resulting in a lower functional reserve that creates earlier susceptibility. The defect may also relate to immunological memory (acquired immune system) rather than earlier

immune response[494]. Over the last four decades, the incidence of diarrhea, malaria, and bronchiolitis decreased by 80% in those under 1 year of age in The Gambia[327]; however, incidence of pneumonia has increased over time.

As previously described, the rural Gambian environment also creates a setting with high food insecurity. Suboptimal dietary intake can result in a myriad of adverse health consequences, including increasing susceptibility to infection. This connection can fall into a cycle that can result in growth stunting[73,126,495]. Moore (2016) reports that The Gambia's strong seasonality creates a setting in which month of birth is a strong proxy of nutrition, infectious diseases, and mortality in early life. This tight relationship may suggest that early life nutrition-related or unrelated infections may permanently damage components of human immune function in these populations[216]. Over 5 decades of consistent collection of demographic, morbidity, and mortality data (along with many others) in this region show that the majority of deaths with documented causes were linked to infectious etiology[216,491]. Moore et al. (2006) explain that long-term effects on immune programming in these Gambian populations likely have a nutritional-origin.

Maternal milk and infant immune function

Immune programming is also likely shaped by bioactive factors in mother's milk, concentrations of which have been shown to be seasonally influenced[280,334,406,437,496]. The mixed effects model and multiple linear regression model analyses reported in Chapter 3 did not show a significant seasonal effect on relative abundances of milk lactoferrin or IgA. Other studies in West Kiang populations found that milk IgA increased during the wet season, suggesting possible environmental pressures on immune system defenses that become evident in maternal milk composition[178,406]. TRP content was influenced by environmental conditions in the milk analysis subset of HERO-G mothers (see Chapter 3), with higher concentrations observed in milk produced during the dry season. This may reflect shifts in maternal energetic resources during periods of food insecurity and increased disease burden.

The present analysis found no apparent influence of seasonality on the relative abundance of sialylated, undecorated, or sia-fuc HMO classes. However, milk produced during the dry season significantly predicted lower relative abundance of fucosylated HMO. Davis et al. (2017) found evidence that mothers nursing during the wet season produced significantly lower abundances of total HMO relative to those breastfeeding during the dry season. These results both indicate a seasonal impact, though the directionality cannot be directly compared as total HMO includes abundance of all HMO classes and fucosylated HMO represents a single class. Evidence from other studies suggests that increased total HMO specifically is associated with infant health outcomes. Milk true protein was particularly

sensitive to environmental conditions (see Chapter 3), perhaps reflecting shifts in maternal energetic resources during periods of food insecurity and increased disease burden.

Secretor status and infant health outcomes

Lewis et al. (2015) report that infants breastfed by non-Secretor mothers experience a delay in bifidobacteria colonization[497]. *Bifidobacterium* species are uniquely equipped to consume HMOs in maternal milk, and there is evidence that they are uniquely beneficial to infant health and development in numerous ways[191,498–502]. HMO composition, determined largely by maternal genetics (Secretor status), plays a critical role in shaping the infant gut microbiota[503]. This influence persists beyond the breastfeeding period. In fact, maternal Secretor status has an observed impact on gut microbiota at 2 to 3 years of age in those exclusively breastfed to at least 4 months of age[503]. Additionally, maternal Secretor status significantly affected Rotavirus vaccination performance in a cohort of Bangladeshi infants[454]. This effect was more apparent in Secretor compared to non-Secretor infants, suggesting that the combination of mother-infant Secretor statuses may be important in determining health outcomes.

Socioeconomic position and infant health

In low-income countries, populations living in high socioeconomic position (SEP) are more likely to have access to adequate health care with care standards comparable to those in high-income countries[125]. Thus, populations most likely to face barriers in access to health and nutrition interventions or other resources are those in resource-poor settings, such as those in rural Gambia.

Household crowding (>1 person per room in a household dwelling) is a risk factor in a variety of infectious diseases. In the Greater Banjul and Upper River region of The Gambia, strong evidence suggests an association between bed-sharing with someone with a cough and severe and non-severe pneumonia[504]. In Chapter 2, an assessment of household SEP in the HERO-G subsample showed that 87.3% of study participants lived in crowded households and many (61.4%) were of low SEP status (see Chapter 2 for methodology and questionnaire results). The calculation used to assess household SEP takes multiple variables into consideration at once, which is more appropriate here than using variables such as maternal education or livestock ownership – common factors incorporated into wealth or asset score measurements – as single predictor variables.

The Medical Research Council (MRC) has made considerable investments in healthcare and nutrition-related infrastructure in their core study villages in the rural region of the West Kiang; In the core study villages in the rural region of the West Kiang, communities have access to ante- and postnatal care, primary health care clinics, all children are vaccinated and receive many WHO recommended health interventions (e.g., vitamin A and mebendazole), growth monitoring during early life, treatment for severely malnourished children, among many other resources. Records show that around 1,500 patients are seen at the MRC Keneba field station clinic annually[246]. Clean piping for water supply has been installed in all compounds, which prevents health issues related to contaminated water. Universal primary education is free in these communities, with enrollment around 97%. The robust resource investments and continued individual and community-level healthcare creates a unique context, as government-implementation of such efforts throughout low-income countries is challenging due to required associated costs.

RESEARCH AIMS

Aims

In this Chapter, I detail the occurrences of infant morbidity in the HERO-G subsample during the first 12 months of life in order to: 1) characterize early life health status in this subsample; and 2) assess possible effects of infant diet on morbidity during infancy and early childhood. Specifically, I investigate relationships between number of infant morbidity occurrences, maternal milk composition (macronutrients, HMOs, HMGPs), and exclusive breastfeeding duration, taking into consideration the potential influences of demographic (household SEP) maternal (parity, Secretor status), infant (sex) and environmental factors (birth season) at 3, 6, 9, and 12 months of age. To investigate possible extended effects of early life diet on health outcomes, cumulative infant morbidity at 9 and 12 months of age were assessed in relationship to exclusive breastfeeding status and maternal milk composition at 3 and 6 months of age.

METHODS

Sample size

The HERO-G subsample (N=194) (infants who were designated exclusive breastfeeding durations and characterized in Chapter 2) was utilized for the present analysis of relationships between diet and morbidity occurrence.

Infant feeding practices

Dietary questionnaires regarding infant feeding were administered to mothers by trained field workers every 10 days starting at one week of infant age until 12 months of infant age. Infant feeding practice was defined by exclusive breastfeeding status at 6 months of age, based on the WHO recommended exclusive breastfeeding duration of 6 months of age[321]. Infants were categorized as either 'EBF <6mo' (provision of breast milk and non-breast milk foods/liquids before 6mo) or 'EBF ≥6mo' (provision of breast milk only until 6 months or later)[264,322]. Additional details regarding infant dietary questionnaire methodology can be found in Chapter 2.

Maternal milk composition

Concentrations of four macronutrients (fat, protein, lactose, true protein) and the relative abundances of four human milk oligosaccharide classes (fucosylated, sialylated, undecorated, sialylated and fucosylated [sia-fuc]) and two human milk glycoproteins (lactoferrin, IgA) were analyzed in milk collected from a subset of 50 mothers in the larger HERO-G cohort at 3, 6, 9, and 12 months of lactation following the methodologies described in Chapter 3. Briefly, mid-infrared technology was used to measure concentrations of the four milk macronutrients, and mass spectrometry was implemented to quantify the relative abundances of the four HMO classes and the two HMGPs. Milk intake volumes were not measured in the HERO-G study.

Infant morbidity data

Infant morbidities were diagnosed and recorded by clinicians at the MRC Keneba field station at scheduled clinic visits at 3, 6, 9, 12, 18, and 24 months of age, plus non-scheduled visits (caregivers sought and were provided MRC clinical evaluation and treatment for infant morbidity as needed). This dissertation focuses its primary analysis on cumulative morbidity occurrences reported at 3, 6, 9, and 12 months age. Infant morbidity diagnoses were sorted into umbrella categories based on etiology. The categories include: dermatological, ear, gastrointestinal (GI), hematological, nutritional, ophthalmological, oral, respiratory, and urinary. Diagnoses included under each category are detailed in **Appendix Table A.6.** Cumulative morbidity (defined here as the total number of morbidity occurrences documented at either scheduled or non-scheduled clinic visits within a specified time period) was incorporated as a continuous variable in the analyses.

Distance to MRC clinic

Proximity of living area to the MRC clinic has been shown to influence health-seeking behavior[505]. Because the present analysis of cumulative morbidity occurrence includes both scheduled and unscheduled clinic visits, relationships between village type/distance to clinic and morbidity occurrence were examined to determine potential influences of proximity to the clinic. Linear regressions were constructed to assess these relationships.

Socioeconomic position

Fieldworkers administered a socioeconomic questionnaire during the booking visit for the HERO-G study. Mothers were asked to provide information regarding sociodemographic variables (maternal education attainment), household characteristics (crowding index [number of persons per room within a dwelling], material of dwelling walls and floor), and durable assets (livestock ownership, possession of a cart). Questionnaire responses describing sociodemographic characteristics, household characteristics, and durable assets were used to generate an asset score using Principal Component Analysis (PCA). Complete details on the socioeconomic position (SEP) calculation and categorization are provided in Chapter 2.

Statistical analysis

Descriptive statistics were calculated for overall morbidity occurrences during the first 12 months of life and stratified by age (3, 6, 9, and 12 months). The dietary variables (milk composition and EBF status) at the 3 and 6 month time points – which fall within the period of time where maternal milk is the predominant or sole food in the infant diet in this population – were also evaluated for both "real time" effects and possible "extended" effects of early life nutritional environment infant health outcomes at 9 and 12 months of age. Here, "real time" effects are assessed using dietary conditions (milk composition and EBF status) at output time point in relationship to health outcomes at that same time point (e.g., milk composition at 3 months of lactation and EBF status at 3 months of age in PLS regression assessing infant morbidity occurrence at 3 months of age). "Extended" effects are assessed using dietary conditions (milk composition and EBF status) at earlier time points in relationship to health outcomes at later output time point (e.g., milk composition at 3 months of lactation and EBF status at 3 months of age in PLS regression assessing morbidity occurrence at 12 months of age).

Partial Least Squares (PLS) regression models were constructed to identify maternal, infant, sociodemographic, and environmental factors associated with infant health outcomes. Individual PLS regression models were used to examine both "real time" effects and "extended" effects of early life diet on infant health outcomes. PLS is a regression, classification, dimension reduction technique that has the ability to model relations between sets of observed variables by means of latent variables[506–508]. First described by Wold (1966), PLS works by fitting linear models based on linear combinations, called factors, of the explanatory variables (Xs)[506]. These factors are constructed in a way that attempts to maximize the covariance between the Xs and the response(s) (Ys). In this way, PLS utilizes the correlations between the Xs and Ys to reveal underlying latent structures. The factors can address the goals of explaining response variation and predictor variation. PLS performs well in situations such as the following, where the use of ordinary least squares or classic linear modeling does not produce satisfactory results: 1) where there are more X variables than observations; 2) where there are highly correlated X variables; 3) where there are a large number of X variables; and 4) several Y variables and many X variables[506]. Factors that explain response variation provide good predictive models for new responses, and factors that explain predictor variation are well represented by the observed values of the predictors.

In Chapter 3, maternal milk nutritional and bioactive factors showed some strong correlations (R^2>0.8) with each other across the lactation period. Multicollinearity reduces the precision of the estimated coefficients, which weakens the overall statistical power of a multiple linear regression. One strategy is to reduce the full model by "dropping" one of the highly correlated variables based on the nature of the relationships and evidence from existing literature. However, reducing a model in this way may overlook effects of the explanatory variables that were removed. Because this dissertation aims to provide a comprehensive evaluation of the role of all dietary factors quantified (milk macronutrients, HMOs, HMGPs, and infant exclusive breastfeeding duration), removing certain milk constituents from the models may obscure the results. Additionally, the small sample sizes from the milk composition analysis, particularly at the 3 month collection time point, would result in a greater number of explanatory (X) variables than observations. In this context, PLS regression modeling is a better fit to investigate the research questions in the following Chapters of the dissertation.

Validation Normalization of the observations (values of both X and Y variables) was achieved using mean centering and unit variance scaling. Predictors and the responses are centered and scaled to have mean 0 and standard deviation 1 by default. Centering the predictors and the responses ensures that the criterion for choosing successive

factors is based on how much variation they explain (in either the predictors, responses, or both)[508]. Without centering, both the mean variable value and the variation around that mean are involved in selecting factors. Scaling places all predictors and responses on an equal levels relative to their variation in the dataset. Validation of the PLS model was performed using 10-fold cross-validation. The number of factors, with factor being defined as the combination of explanatory variables, are presented in the Results section of this Chapter. The percentage of variation explained for cumulative Y (% of variation in output variable explained by the factor) indicates how well the explanatory variables can predict the output, and, alternatively the percentage of variation explained for cumulative X (% of variation in explanatory variables explained by the output variable) indicates how well the explanatory variables are predicted by the output variable. Both of these percentages are presented in the Results section of this Chapter, though emphasis is placed on the importance of the percentage of variation explained for cumulative Y. Results of PLS regression models were also assessed and interpreted using the prediction error sum of squares (PRESS) and the Variable Importance of the Projection (VIP) statistic.

PRESS measures the predictive power of the model, providing information about the significance of the component (a component is considered significant when PRESS/Residual sum of squares < 1)[507,508]. Specifically, PRESS measures deviation between the fitted values and the observed values; it is similar to the sum of squares of the residual error (SSE), which is the summation of the squared residuals. However, PRESS uses a different calculation for the residuals. The formula used to calculate PRESS is equivalent to a process of systematically removing each observation from the data set, estimating the regression equation, and determining how well the model predicts the removed observation. An optimum number of factors is identified using the minimum Root Mean PRESS statistic. Generally, the smaller the PRESS value, the better the model's predictive ability. The model with minimum root mean PRESS has the optimal number of factors. The VIP statistic is a weighted sum of squares and it measures a predictor's contribution to characterizing factors used in the PLS model or in defining the projection[506,507]. Cut-off values for the VIP vary throughout the literature, but there is some consensus that VIP values >1.0 indicate predictors that are "important," or most relevant to explaining the dependent variable[508].

Results sections of PLS regression model results include presentation of: (1) number of PLS regression factors, with factor being defined as the combination of explanatory variables; (2) description of the percentage of variation explained for cumulative Y and the percentage of variation explained for cumulative X; (3) assessment of PRESS and the VIP statistic.

Explanatory variables included the PLS regression models included: concentration of macronutrients (g/dL) (fat, protein, lactose, true protein), relative abundances (%) of HMO classes (fucosylated, sialylated, undecorated, and sia-fuc), abundance of HMGPs (lactoferrin, IgA), and EBF duration, and all models were adjusted for infant sex, birth season, maternal parity, SEP score (see Chapter 2 for calculation details), maternal Secretor status, and maternal MUAC during the third trimester of pregnancy. The explanatory and outcome variables included in each PLS regression model are presented in **Tables 4.1-4.2.** Mixed effects models were constructed as a comparison of statistical approaches for assessment of potential predictors of health outcomes during first year of life. The mixed effects models incorporated all of the same explanatory variables as the PLS regression, with the exception of the relative abundance of undecorated HMO, which was dropped due to its high collinearity with fucosylated HMO (see Chapter 2 Results). Additionally, Subject ID was incorporated into the mixed effects model to control for repeated measurements, and collection time point was included to account for variation in lactation stage. Significant associations identified in the mixed effects models were summarized using the beta regression coefficients (B), 95% confidence intervals (CI), and P-values. The level of statistical significance was set to $P < 0.05$ for all analyses. All statistical analyses were conducted using JMP Pro 15.0 statistical software (©2019 SAS Institute, Inc.).

Table 4.1. PLS regression explanatory and outcome variables assessing potential "real time" effects of diet (EBF status and maternal milk composition) on cumulative infant morbidity at 3, 6, 9, and 12 months of age

Outcome variables	Explanatory variables
Cumulative infant morbidity at 3mo of age	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● EBF status at 3mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 3mo (Macronutrients, HMOs, HMGPs)
Cumulative infant morbidity at 6mo of age	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● EBF status at 6mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 6mo (Macronutrients, HMOs, HMGPs)
Cumulative infant morbidity at 9mo of age	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 9mo (Macronutrients, HMOs, HMGPs)
Cumulative infant morbidity at 12mo of age	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 12mo (Macronutrients, HMOs, HMGPs)

[a]Maternal MUAC measurements were collected at 36 weeks of gestation

Table 4.2. PLS regression explanatory and outcome variables assessing potential "extended" effects of diet (EBF status and maternal milk composition) at 3 and 6 months on cumulative infant morbidity at 9 and 12 months of age

Outcome variables	Explanatory variables
Cumulative infant morbidity at 9mo of age (3mo dietary variables)	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● EBF status at 3mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 3mo (Macronutrients, HMOs, HMGPs)
Cumulative infant morbidity at 9mo of age (6mo dietary variables)	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● EBF status at 6mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 6mo (Macronutrients, HMOs, HMGPs)
Cumulative infant morbidity at 12mo of age (3mo dietary variables)	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● EBF status at 3mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 3mo (Macronutrients, HMOs, HMGPs)
Cumulative infant morbidity at 12mo of age (6mo dietary variables)	● Infant sex (F/M) ● Infant birth season (Wet/Dry) ● Socioeconomic position (SEP score; continuous) ● Maternal parity (continuous) ● EBF status at 6mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Maternal milk composition at 6mo (Macronutrients, HMOs, HMGPs)

[a]Maternal MUAC measurements were collected at 36 weeks of gestation

Table 4.3. Response and explanatory variables investigated in mixed effects model

Outcome variable*	Explanatory variable
Cumulative infant morbidity	*Fixed* ● Maternal MUAC[a] (continuous) ● Infant sex (F/M) ● Season of milk collection (wet/dry) ● Infant season of birth (wet/dry) ● Socioeconomic position (SEP score) ● Parity (continuous) ● EBF duration (months) ● Collection time point (3, 6, 9, 12mo) *Random* ● Subject ID

[a]Maternal MUAC measurements were collected at 36 weeks of gestation

RESULTS

Maternal and infant characteristics

A total of 194 maternal-infant pairs were included in the analysis, with a mean (±SD) maternal age of 32.0 (±6.9) years**.** Of the 194 infants, there were a total of 103 male and 91 female infants, with 145 born during the dry ("harvest") season and 49 born during the wet ("hungry") season. Average age at introduction of non-breast milk foods was 5.0 (±1.5) months, with 59 (30.4%) infants EBF ≥6mo and 135 (69.6%) of infants EBF <6mo.

Infant feeding

The mean (±SD) age for introducing any food or liquid other than breast milk was 5.0 (±1.5) months with 135 (69.6%) of infants EBF <6mo and 59 (30.4%) infants EBF ≥6mo. Additional details regarding infant feeding practices in this subsample are described in Chapter 2.

Subsample morbidity profiles

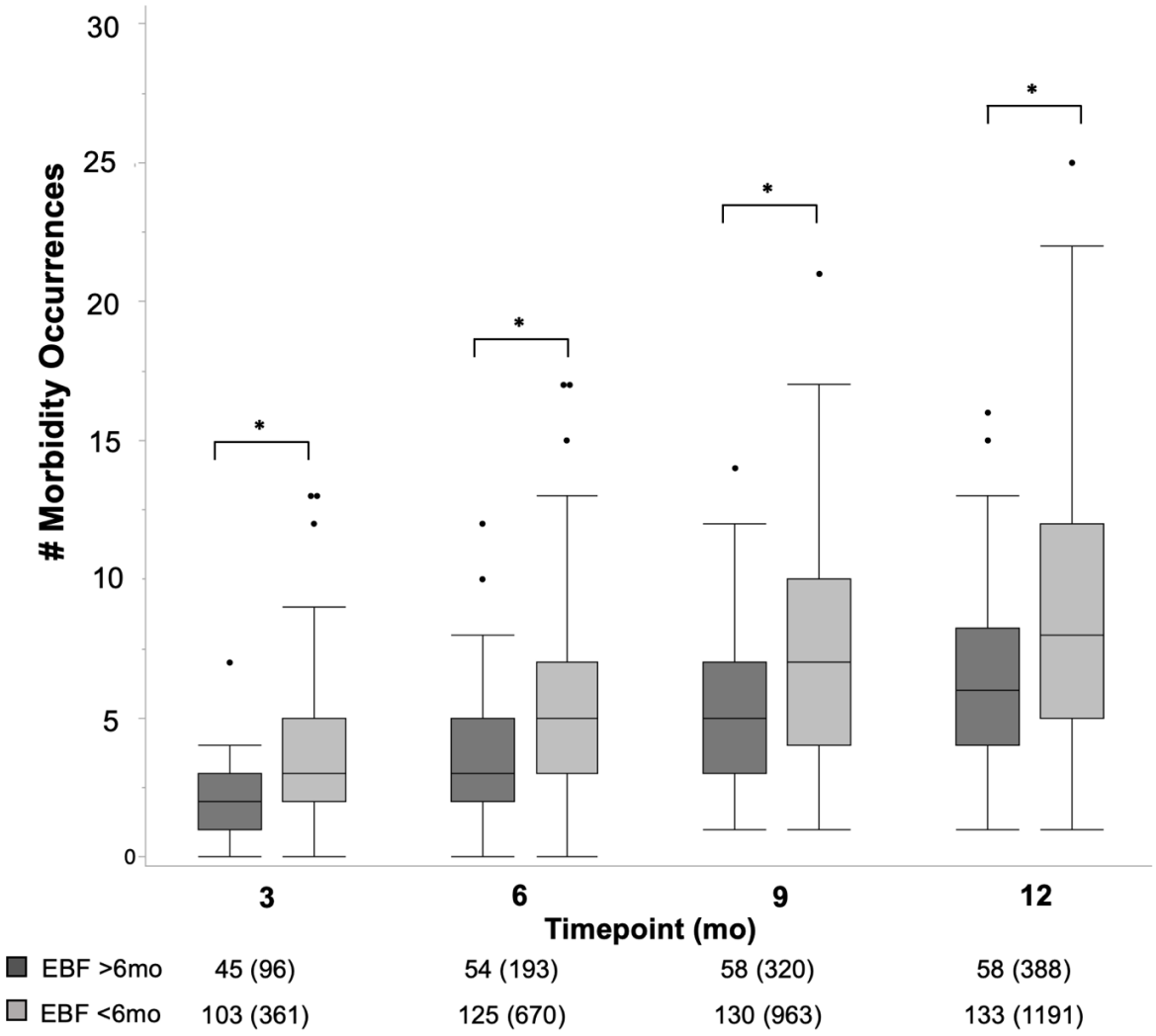
A total of 1,579 morbidities were documented across the first 12 months of life in this subsample (N=194). At 6 months of age, infants EBF <6mo (the threshold for categorization of EBF duration; N=135) experienced 670 morbidity occurrences (mean: 5.36 ±3.2) compared to the 193 cumulative events (mean: 3.57, ±2.3) experienced by infants EBF ≥6 months (N=59) (**Table 4.4).** By 12 months of age, the number of morbidity occurrences increased to 1,191 (mean: 8.95, ±4.8) in infants EBF <6mo and 388 (mean: 6.69, ±3.6) in infants EBF ≥6mo. The average number of morbidity occurrences was significantly higher at 3 (P=0.0029), 6 (P=0.0002), 9 (P=0.0021), and 12 (P=0.0018) months of age for infants EBF <6mo compared to infants EBF ≥6 months. **Figure 4.1** depicts a comparison of morbidity occurrence between infants EBF 6mo (N=135) and ≥6mo (N=59) at 3, 6, 9, and 12 months of age. **Appendix Table A.7** details morbidity occurrence distributions in the milk analysis subset.

Table 4.4. Cumulative morbidity at 3, 6, 9 and 12mo of life according to sex, birth season, parity, and EBF duration

Age (mo)	Value	Female (N=91)	Male (N=103)	Dry (N=145)	Wet (N=49)	Multiparous (N=177)	Primiparous (N=17)	EBF <6mo (N=135)	EBF ≥6mo (N=59)
3	Mean	2.97	3.20	2.74	4.11	2.97	**4.31***	**3.50****	2.13
	SD	2.27	2.64	2.21	2.89	2.48	2.06	2.70	1.42
	N (infants)	72	76	110	38	135	13	103	45
	Sum (morbidity)	214	243	301	156	401	56	361	96
6	Mean	4.61	5.01	4.62	5.48	4.64	**6.80***	**5.36****	3.57
	SD	2.54	3.48	2.89	3.58	2.92	4.04	3.22	2.26
	N (infants)	84	95	137	42	164	15	125	54
	Sum (morbidity)	387	476	633	230	761	102	670	193
9	Mean	6.61	7.01	6.82	6.83	6.61	**9.33***	**7.41****	5.51
	SD	3.27	4.42	3.84	4.21	3.81	4.48	4.11	3.11
	N (infants)	88	100	142	46	173	15	130	58
	Sum (morbidity)	582	701	966	314	1143	140	963	320
12	Mean	8.04	8.46	8.09	8.79	8.10	10.13	**8.95****	6.69
	SD	3.88	5.12	4.44	4.97	4.50	5.14	4.81	3.56
	N (infants)	89	102	143	48	175	16	133	58
	Sum (morbidity)	716	863	1157	422	1417	162	1191	388

*P<0.05; **P<0.01; A variable with a significant difference (indicated by boldface font and p-value<0.05 or 0.01) indicates a significantly *greater* number of morbidity occurrences relative to its comparative group (e.g., a boldface p-value in the EBF <6mo column indicates that infants EBF <6mo experienced significantly more morbidity occurrences compared to infants EBF ≥6mo).

Figure 4.1. Comparison of morbidity occurrence between infants EBF <6mo (N=135) and ≥6mo (N=59) at 3, 6, 9, and 12 months of age



Data presented as N (sum) [N: number of infants with any reported morbidity; sum: total number of morbidity reports by respective time point]; *P<0.05; **P<0.01

Of the total morbidity reports documented over the first 12 months of life, the most commonly occurring morbidities were of respiratory (57.4%), dermatological (21.2%), and gastrointestinal (10.2%) origin. There was no significant difference between occurrence of specific morbidity types based on infant birth season, sex, or EBF duration **(Table 4.5).** Additionally, there were no significant differences in infant morbidity occurrence based on village proximity to the MRC clinic at 3, 6, or 9 months of age (P>0.05). However, there was a significant inverse relationship between total morbidity occurrences and distance to clinic at 12 months of age (P=0.0089). As such,

distance to clinic will be incorporated as a random variable in the mixed effects model, which assesses morbidity across the entire first year of life.

Table 4.5. Summary statistics of reported infant morbidities between birth and 12 months of age according to infant sex, season of birth, and feeding practice

Morbidity Type	**Total # of reports**	**Sex**		**Birth Season**		**EBF Duration**	
		F (N=91)	**M** (N=103)	**Dry** (N=130)	**Wet** (N=64)	**<6mo** (N=135)	**≥6mo** (N=59)
Dermatological	516	249	267	384	132	379	137
Ear	37	14	23	27	10	24	13
GI	248	125	123	185	63	163	85
Respiratory	1401	614	787	1031	370	1086	315
Nutritional	10	1	9	3	7	5	5
Ophthalmological	79	34	45	54	25	55	24
Hematological	60	22	38	39	21	43	17
Oral	65	27	38	52	13	35	30
Urinary	26	17	9	20	6	19	7
Total	2442	1103	1339	1795	647	**1809***	633

*P<0.05; A variable with a significant difference (indicated by boldface font and p-value<0.05 or 0.01) indicates a significantly *greater* number of morbidity occurrences relative to its comparative group (e.g., a boldface p-value in the EBF <6mo column indicates that infants EBF <6mo experienced significantly more morbidity occurrences compared to infants EBF ≥6mo).

PLS regression results (predictors of cumulative morbidity)

Health outcomes at 3 months of age

In the PLS regression for the 3 month time point, the minimum root PRESS, the measure of the predictive power of the model, was 0.9812, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 40.6% and the percent variation explained for cumulative Y was 79.1%. The 1-component model showed 5 influential variables (VIP > 1.0) on infant morbidity occurrence at 3 months of age.

Exclusive breastfeeding status at 3 months of age had the strongest influence on infant morbidity occurrence at 3 months of age (VIP: 2.0186). Infants no longer EBF at 3 months of age were predicted to experience a greater number of cumulative morbidity occurrences relative to those still EBF at that time (B: 0.2084 morbidity occurrences). Birth season also influenced morbidity occurrence; being born during the wet ("hungry") season predicted a greater number of cumulative infant morbidities by 3 months of age relative to those born in the dry ("harvest") season (VIP:

1.7131; B: 0.1768 morbidity occurrences). Greater parity had a positive correlation with morbidity occurrence at 3 months, with higher parity predicting a greater number of morbidities by 0.1409 morbidity occurrences (VIP: 1.3652). Milk HMO composition was also associated with infant morbidity occurrence, with greater relative abundance of the fucosylated HMO class structures predicting fewer morbidities (VIP: 1.979; B: -0.1340 morbidity occurrences) and greater relative abundance of undecorated HMO class structures predicting a greater number of morbidities (VIP: 1.0886; B: 0.1124 morbidity occurrences). None of the other variables included in the PLS regression were influential.

Health outcomes at 6 months of age

In the PLS regression for the 6 month time point, the minimum root PRESS was 0.9920, which is considered significant, and the minimizing number of factors was 1. The percent of variation explained for cumulative and Y was 52.0% and 70.4%, respectively. The 1-component model showed 6 influential variables (VIP > 1.0) on infant morbidity occurrence at 6 months of age.

Lactose content had the strongest influence on infant morbidity, with greater concentrations of lactose associated with fewer morbidity occurrences (VIP: 1.9913; B: -0.1286 morbidity occurrences). Next, relative abundance of milk IgA expressed at 6 months of lactation had a strong relationship with infant morbidity occurrence, with greater IgA having a positive relationship with morbidity (VIP: 1.4676; B: 0.0948 morbidity occurrences). Relative abundance of sia-fuc HMO class had a positive relationship with infant morbidities (VIP: 1.3521; B: 0.0873 morbidity occurrences). TRP content had a positive association with infant morbidity occurrence at 6 months (VIP: 1.3181; B: 0.0851 morbidity occurrences). EBF at 6 months was associated with fewer morbidities (VIP: 1.0789; -0.0697). Greater parity was associated with a greater number of infant morbidities by 0.0713 morbidity occurrences (VIP: 1.1039). None of the other variables were considered influential in the PLS regression.

Health outcomes at 9 months of age

"Real time" effects

In the PLS regression for the 9 month time point, the minimum root PRESS was 0.9975, which is considered significant, and the minimizing number of factors was 1. The percent of variation explained for cumulative and Y was 41.5% and 51.8%, respectively. The 1-component model showed 7 influential variables (VIP > 1.0) on infant morbidity occurrence at 9 months of age.

Higher maternal parity had the strongest influence on was positively associated with infant morbidity occurrence (VIP: 1.9457; B: 0.1722). Maternal nutrition during pregnancy also had a positive relationship with morbidity occurrence, with higher MUAC during the 3rd trimester linked with higher morbidity occurrences by 0.0989 morbidities (VIP: 1.1181). Greater relative abundance of fucosylated HMO had a negative relationship with infant morbidity occurrence (VIP: 1.7403; B: -0.1540), but a positive relationship with sialylated (VIP: 1.1782; B: 0.1042) and undecorated (VIP: 1.6971; B: 0.1502). Protein content was also important in predicting infant morbidity, with greater protein content positive associated with infant morbidity occurrence by 0.0908 morbidity occurrences (VIP: 1.0267). Lactose had a negative influence on morbidity, with greater lactose content predicting fewer infant morbidity occurrences (VIP: 1.1892; B: -0.1052). None of the other variables were considered influential in the PLS regression.

"Extended" effects of dietary variables at 3 months of age

PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant health outcomes at 9 months of age had a minimum root PRESS was 0.9767 and the minimizing number of factors was 2. The percent of variation explained for cumulative and Y was 25.1% and 47.3%, respectively. The 1-component model showed 5 influential variables (VIP > 1.0) on infant morbidity occurrence at 9 months of age.

Maternal parity had the strongest influence on morbidity occurrence at 9 months of age (VIP: 1.5846; B: 0.1943). Relative abundance of HMO classes also had strong influence on infant morbidity occurrence at 9 months of age. Greater relative abundance of fucosylated HMO at 3 months had a negative association with morbidity occurrence at 9 months of age (VIP: 1.4682; B: -0.1280). Greater relative abundances of sialylated and undecorated HMO classes at 3 months had positive associations with morbidity occurrence at 9 months (sialylated HMO - VIP: 1.0056; B: 0.0821; undecorated HMO – VIP: 1.4209; B: 0.1296). EBF status at 3 months had a negative association with infant morbidity at 9 months of age, where infants still EBF at 3 months predicted fewer morbidity occurrences by -0.1488 occurrences (VIP: 1.4093). Finally, maternal secretor status was negatively associated with infant morbidity occurrence, with infants of mothers categorized as non-secretors at 3 months of lactation predicting fewer infant morbidity occurrences at 9 months of age by -0.1601 (VIP: 1.0593). None of the other variables were considered influential in the PLS regression.

"Extended" effects of dietary variables at 6 months of age

PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant health outcomes at 9 months of age had a minimum root mean PRESS of 0.9874, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 37.2% and 45.7% for the percentage of variation explained for cumulative Y. The 1-component model showed 5 influential variables (VIP > 1.0) on infant morbidity occurrence at 9 months of age.

Maternal parity had the strongest influence on infant morbidity at 9 months of age (VIP: 1.9817; B: 0.1667). Relative abundance of fucosylated HMO at 6 months of lactation had a negative association with infant morbidity at 9 months of age, with greater relative abundance of fucosylated HMO predicting fewer morbidity occurrences by -0.1491 occurrences (VIP: 1.7725). Greater lactose content at 6 months of lactation had a negative influence on infant morbidity occurrence at 9 months of age (VIP: 1.2113; B: -0.1019), and greater protein content had a positive association with morbidity occurrence (VIP: 1.0457; B: 0.0880). Finally, maternal nutritional status during the 3rd trimester of pregnancy had a positive association with infant morbidity, with higher MUAC measurements predicting a greater number of morbidities by 0.0958 occurrences (VIP: 1.1388). EBF status at 6 months did not have a strong influence on morbidity occurrence at 9 months of age (VIP: 0.8031). None of the other variables were considered influential in the PLS regression.

Health outcomes at 12 months of age

"Real time" effects

In the PLS regression for the 12 month time point, the minimum root PRESS was 0.9928, which is considered significant, and the minimizing number of factors was 1. The percent of variation explained for cumulative and Y was 33.5% and 52.4%, respectively. The 1-component model showed that relative abundance of sialylated and sia-fuc HMO, lactose, parity, protein, IgA, and birth season were influential (VIP > 1.0) on infant morbidity occurrence by 12 months of age.

Relative abundance of sialylated HMO at 12 months had the strongest influence on infant morbidity occurrence, with greater relative abundance associated with greater number of morbidity occurrences by 0.1140 (VIP: 1.7736). Relative abundance of sia-fuc HMO also had a strong positive association with infant morbidity occurrence at 12 months (VIP:1.6372; B: 0.1052). Higher lactose content predicted fewer infant morbidity occurrences by -0.1039

morbidities (VIP: 1.6176). Next, higher maternal parity predicted greater number of morbidity occurrences at 12 months by 0.0961 morbidities (VIP: 1.4964). Milk protein and IgA were both positively associated with morbidity occurrence, with greater milk protein predicting a greater number of morbidity occurrences by 0.0732 morbidities (VIP: 1.1386) and greater relative abundance of milk IgA predicting a greater number of morbidity occurrences by 0.0645 morbidities (VIP: 1.004). Finally, birth season had an important influence on infant morbidity at 12 months, with a positive relationship between birth during the wet season and number of morbidity occurrences (VIP: 1.0175; B: 0.0654). None of the other variables were considered influential in the PLS regression.

"Extended" effects of dietary variables at 3 months of age

PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant health outcomes at 12 months of age showed several influential variables. The minimum root mean PRESS was 0.9714 and the minimizing number of factors was 1. The percent variation explained for cumulative X was 40.4% and 88.0% for the percentage of variation explained for cumulative Y. The 1-component model showed 5 influential variables (VIP > 1.0) on infant morbidity occurrence at 12 months of age.

EBF status at 3 months of age had the strongest influence on infant morbidity occurrence at 12 months of age, with infants still EBF at 3 months predicted to experience fewer morbidities by -0.1378 occurrences (VIP: 1.8195). Greater relative abundances of sialylated and sia-fuc HMO at 3 months of lactation had positive associations with infant morbidity at 12 months (sialylated HMO – VIP: 1.5429; B: 0.1169; sia-fuc HMO – VIP: 1.4242; B: 0.1079). Higher lactose content in maternal milk at 3 months of lactation predicted fewer infant morbidities at 12 months of age by -0.1066 occurrences (VIP: 1.4072). Finally, higher maternal parity had a positive association with infant morbidity at 12 months of age (VIP: 1.3017; B: 0.0986). None of the other variables were considered influential in the PLS regression.

"Extended" effects of dietary variables at 6 months of age

PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant health outcomes at 12 months of age had a minimum root mean PRESS of 0.9902, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative

X was 40.4% and 78.0% for the percentage of variation explained for cumulative Y. The 1-component model showed 7 influential variables (VIP > 1.0) on infant morbidity occurrence at 3 months of age.

Relative abundance of sialylated HMO at 6 months of lactation had the strongest influence on infant morbidity at 12 months of age (VIP: 1.8005; B: 0.1132). Sia-fuc HMO relative abundance at 6 months of lactation also positively influenced infant morbidity at 12 months of age, with greater relative abundance of this HMO class predicting a greater number of morbidities by 0.1045 occurrences VIP: 1.6620). Lactose content at 6 months of lactation negatively influenced infant morbidity occurrence at 12 months of age (VIP: 1.6422; B: -0.1032). Greater maternal parity was associated with greater number of morbidity occurrences at 12 months (VIP: 1.5191; B: 0.0955). Milk protein content and relative abundance of IgA both had positive associations with infant morbidity occurrence (protein – VIP: 1.1559; B: 0.0727; IgA – VIP: 1.0193; B: 0.0641). Being born during the dry season predicted fewer morbidities at 12 months of age by -0.0649 occurrences (VIP: 1.0329). EBF status at 6 months of age did not have a strong influence on infant morbidity occurrence at 12 months of age (VIP: 0.8421).

Table 4.6. VIP statistics and model coefficients (B) from PLS regressions assessing "real time" and "extended" predictors of infant morbidity occurrence at 3, 6, 9, and 12 months of age for centered and scaled data

	"Real time" predictors of morbidity occurrence							
Time Point	**3mo**		**6mo**		**9mo**		**12mo**	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.0066	0.0007	0.3542	-0.0229	0.7229	-0.064	0.4427	-0.0284
Sex[M]	0.0066	-0.0007	0.3542	0.0229	0.7229	0.064	0.4427	0.0284
BirthSeason[Dry]	**1.7131**	-0.1768	0.7046	-0.0455	0.5571	-0.0493	**1.0175**	-0.0654
BirthSeason[Wet]	**1.7131**	0.1768	0.7046	0.0455	0.5571	0.0493	**1.0175**	0.0654
SEP Score	0.1933	0.0200	0.0447	-0.0029	0.3510	-0.0311	0.0062	-0.0004
Parity	**1.3652**	0.1409	**1.1039**	0.0713	**1.9457**	0.1722	**1.4964**	0.0961
Maternal MUAC	0.7062	0.0729	0.5215	0.0337	**1.1181**	0.0989	0.7213	0.0463
SecretorStatus[Non-Secretor]	0.2423	0.0250	**1.4281**	0.0922	0.5784	-0.0512	0.9842	0.0632
SecretorStatus[Secretor]	0.2423	-0.0250	**1.4281**	-0.0922	0.5784	0.0512	0.9842	-0.0632
EBF Status @3mo [Y]	**2.0186**	-0.2084	-	-	-	-	-	-
EBF Status @3mo [N]	**2.0186**	0.2084	-	-	-	-	-	-
EBF Status @6mo [Y]	-	-	**1.0789**	-0.0697	-	-	-	-
EBF Status @6mo[N]	-	-	**1.0789**	0.0697	-	-	-	-
Fat	0.3393	0.035	0.5845	0.0377	0.1199	-0.0106	0.8235	0.0529
Protein	0.2699	0.0279	0.9847	0.0636	**1.0267**	0.0908	**1.1386**	0.0732
Lactose	0.4367	-0.0451	**1.9913**	-0.1286	**1.1893**	-0.1052	**1.6176**	-0.1039
True Protein	0.7355	0.0759	**1.3181**	0.0851	0.9743	0.0862	0.0196	0.0013
Fucosylated HMO	**1.2979**	-0.134	0.1022	0.0066	**1.7403**	-0.1540	0.2727	-0.0175
Sialylated HMO	0.7620	0.0787	0.8256	0.0533	**1.1782**	0.1042	**1.7736**	0.1140
Undecorated HMO	**1.0886**	0.1124	0.3238	-0.0209	**1.6971**	0.1502	0.0927	0.0060
Sia-fuc HMO	0.1658	-0.0171	**1.3521**	0.0873	0.5604	-0.0496	**1.6372**	0.1052
Lactoferrin	0.1968	-0.0203	0.2524	0.0163	0.6748	-0.0597	0.5770	0.0371
IgA	0.1671	-0.0173	**1.4676**	0.0948	0.0864	-0.0076	**1.0040**	0.0645
	"Extended" predictors of morbidity occurrence							
Time Point	**9mo_3mo**		**9mo_6mo**		**12mo_3mo**		**12mo_6mo**	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.6411	-0.0984	0.7363	-0.0620	0.3851	-0.0292	0.4494	-0.0283
Sex[M]	0.6411	0.0984	0.7363	0.0620	0.3851	0.0292	0.4494	0.0283
BirthSeason[Dry]	0.8987	0.0303	0.5674	-0.0477	0.8851	-0.0671	**1.0329**	-0.0649
BirthSeason[Wet]	0.8987	-0.0303	0.5674	0.0477	0.8851	0.0671	**1.0329**	0.0649
SEP Score	0.3321	-0.0528	0.3575	-0.0301	0.0054	-0.0004	0.0063	-0.0004
Parity	**1.5846**	0.1943	**1.9817**	0.1667	**1.3017**	0.0986	**1.5191**	0.0955
Maternal MUAC	0.9937	0.1527	**1.1388**	0.0958	0.6274	0.0475	0.7322	0.0460
SecretorStatus[Non-Secretor]	**1.0593**	-0.1601	0.5891	-0.0496	0.8562	0.0649	0.9991	0.0628
SecretorStatus[Secretor]	**1.0593**	0.1601	0.5891	0.0496	0.8562	-0.0649	0.9991	-0.0628
EBF Status @3mo [Y]	**1.4093**	-0.1488	-	-	**1.8195**	-0.1378	-	-
EBF Status @3mo [N]	**1.4093**	0.1488	-	-	**1.8195**	0.1378	-	-
EBF Status @6mo [Y]	-	-	0.8031	-0.0676	-	-	0.8421	-0.0529
EBF Status @6mo[N]	-	-	0.8031	0.0676	-	-	0.8421	0.0529
Fat	0.2772	0.0166	0.1221	-0.0103	0.7163	0.0543	0.836	0.0526
Protein	0.8662	0.1251	**1.0457**	0.0880	0.9905	0.0750	**1.1559**	0.0727
Lactose	0.9682	-0.1162	**1.2113**	-0.1019	**1.4072**	-0.1066	**1.6422**	-0.1032
True Protein	0.8121	0.1142	0.9923	0.0835	0.017	0.0013	0.0199	0.0012
Fucosylated HMO	**1.4682**	-0.1280	**1.7725**	-0.1491	0.2372	-0.018	0.2768	-0.0174
Sialylated HMO	**1.0056**	0.0821	**1.2000**	0.1010	**1.5429**	0.1169	**1.8005**	0.1132

Undecorated HMO	**1.4209**	0.1296	**1.7286**	0.1454	0.0806	0.0061	0.0941	0.0059
Sia-fuc HMO	0.6791	-0.1097	0.5708	-0.0480	**1.4242**	0.1079	**1.6620**	0.1045
Lactoferrin	0.7281	-0.1181	0.6873	-0.0578	0.502	0.0380	0.5858	0.0368
IgA	0.3689	-0.0480	0.0880	-0.0074	0.8734	0.0662	**1.0193**	0.0641

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) explanatory variables of interest (e.g., 9mo_3mo = 9mo total morbidity occurrence in relationship to 3mo dietary variables); Undec: Undecorated HMO class; Fuc: Fucosylated HMO class; Sia: Sialylated HMO class; Maternal MUAC: 3rd trimester maternal MUAC measurement; VIP: Variable Importance of Projection; B: Beta coefficient

Table 4.7. Summary of important (VIP > 1.0) PLS regression results ("real time" positive and negative predictors) of infant health outcomes at 3, 6, 9, and 12 months of age for centered and scaled data

3mo		6mo		9mo		12mo	
Positive	***Negative***	***Positive***	***Negative***	***Positive***	***Negative***	***Positive***	***Negative***
Birth season (wet) Parity EBF @ 3mo (N) Undec HMO	Birth season (dry) EBF @ 3mo (Y) Fuc HMO	Parity Non-Secretor EBF @ 6mo (N) TRP Sia-Fuc HMO IgA	Secretor EBF @ 6mo (Y) Lactose	Parity Maternal MUAC Protein Sia HMO Undec HMO	Lactose Fuc HMO	Birth season (wet) Parity Protein Sia HMO Sia-Fuc HMO IgA	Birth season (dry) Lactose

Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Undec: Undecorated HMO class; Fuc: Fucosylated HMO class; Sia: Sialylated HMO class

Table 4.8. Summary of important (VIP > 1.0) PLS regression results ("extended" positive and negative predictors) of diet (EBF status and maternal milk composition) at 3 and 6 months of age on 9 and 12 month health outcomes

9mo_3mo		9mo_3mo		12mo_3mo		12mo_6mo	
Positive	***Negative***	***Positive***	***Negative***	***Positive***	***Negative***	***Positive***	***Negative***
Parity Secretor EBF @ 3mo (N) Sia HMO Undec HMO	Non-Secretor EBF @ 3mo (Y) Fuc HMO	Parity Maternal MUAC Protein Lactose Sia HMO Undec HMO	Fuc HMO	Parity EBF @ 3mo (N) Sia HMO Sia-Fuc HMO	EBF @ 3mo (Y) Lactose	Birth season (wet) Parity Protein Sia HMO Sia-fuc HMO IgA	Birth season (dry) Lactose

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) explanatory variables of interest (e.g., 9mo_3mo = 9mo total morbidity occurrence in relationship to 3mo dietary variables); Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; SeStatus: Maternal Secretor status; Undec: Undecorated HMO class; Fuc: Fucosylated HMO class; Sia: Sialylated HMO class; Maternal MUAC: 3rd trimester maternal MUAC measurement

Mixed effects model results

Maternal milk composition was a significant predictor of infant morbidity occurrence across the first 12 months of life in the mixed effects model. Specifically, higher fat content in milk predicted a greater number of infant morbidities by 0.46 occurrences (95% CIs: 0.11, 0.82 occurrences; P=0.0113). A greater relative abundance of sia-fuc HMO significantly predicted fewer infant morbidities by -1.6 occurrences (95% CIs: -2.37, -0.82 occurrences; P<0.0001). Maternal parity was also a significant predictor of infant morbidity occurrence. Higher parity (by 1 offspring) significantly predicted a greater number of morbidities by 0.76 occurrences (95% CIs: 0.13, 1.40 occurrences; P=0.0202). Finally, the random variable, Subject ID, significantly predicted higher infant morbidity

occurrence (B: 15.32 occurrences; 95% CIs: 7.25, 23.39 occurrences; P=0.0002). Full model results are detailed in **Table 4.9**.

Table 4.9. Mixed effects model results of potential predictors of cumulative morbidity across the first 12 months of life

	Term	B	LB, UB	P
Fixed Effects	Intercept	0.74	-18, 19.48	0.9374
	Sex[F]	-0.90	-2.26, 0.47	0.1919
	BirthSeason[Dry]	0.32	-1.38, 2.01	0.7084
	SEP Score	0.22	-0.28, 0.71	0.3854
	Parity	0.76	0.13, 1.40	**0.0202***
	Maternal MUAC	0.13	-0.45, 0.72	0.6458
	SecretorStatus[Non-Secretor]	-0.22	-1.27, 0.83	0.6772
	EBF duration (mo)	-1.10	-1.98, -0.23	**0.0149***
	Fat	0.46	0.11, 0.82	**0.0113***
	Protein	1.32	-2.54, 5.17	0.4992
	Lactose	-0.15	-1.06, 0.77	0.7493
	True Protein	-0.11	-4.19, 3.96	0.9572
	Fucosylated	0.07	-0.01, 0.15	0.0690
	Sialylated	-0.24	-0.74, 0.26	0.3452
	Sialofucosylated	-1.60	-2.37, -0.82	**<.0001****
	Lactoferrin	-0.66	-2.6, 1.28	0.5030
	IgA	1.30	-2.49, 5.10	0.4977
Random Effects	SubjectID	15.32	7.25, 23.39	**0.0002****
	Proximity to clinic	2.75	-4.72, 18.35	0.5150

B: Coefficient estimate; LB, UB: 95% CI's (Lower Bound, Upper Bound); P: P=P-value; *P<0.05; **P<0.01

DISCUSSION

Infant health and development can be shaped by the complex cyclical interactions between nutrition and infection in early life[361,463,464]. Infants are at high risk of infection during early life while their immune systems mature, and leading causes of infant mortality are due to commonplace morbidities that arise from infection[509]. In The Gambia, high rates of under 5 mortality from diarrhea and pneumonia peak during complementary feeding age. Here, infant health outcomes were assessed in relationship to infant diet (maternal milk composition and exclusive breastfeeding duration) in the HERO-G subsample. Two of the most frequently reported morbidities in this subsample, respiratory and GI illnesses, are classified by the WHO as the leading causes of death in children under 5 years of age around the world[509]. High occurrence of dermatological morbidities, as was found here, is common in low-income countries, and may relate to components of the skin innate immune system, dietary factors, or environmental conditions[510,511]; it is a common manifestation of chronic inflammation.

Two types of statistical models were constructed to evaluate potential predictors of infant morbidity occurrence: PLS regression models and mixed effects models. Mixed effects models are particularly useful in settings where repeated measures are made on the same statistical units, as is the case in longitudinal studies such as the HERO-G study. This statistical approach is also useful when working with datasets with fixed and random effects, which allows analyses to account for both within person and across person variability. In the context of understanding the nuanced contributions and natural biological variation in health outcomes in relationship to maternal milk composition, a mixed effects model approach is effective. By utilizing PLS regression models, the statistical analysis can handle inclusion of highly colinear variables, which was noted amongst milk constituents, particularly fucosylated and undecorated HMO classes. Removal of relative abundance of the undecorated HMO class from the mixed effects models showed a significant improvement in model fit due to its strong collinearity with the fucosylated HMO class. However, undecorated HMOs have been shown to be a preferred growth substrate by certain species of bifidobacteria[421] and have important anti-microbial properties associated with immunological protection[421]. Thus, utilizing PLS regression allowed for the evaluation of individual contributions by each milk constituent. Results from both model types are discussed below.

PLS regression analyses showed that EBF status at 3 months of age was important in predicting infant morbidity occurrence in "real time" (i.e., at 3 months of age) and showed an "extended" effect on health outcomes at 9 and 12 months. EBF status at 6 months of age was an important predictor of infant morbidity occurrence at 6 months

of age but did not have an important influence in the extended effects PLS regressions on health outcomes at 9 and 12 months of age. This suggests that EBF duration to 3 months is a stronger driver of health outcomes in later life than EBF duration to 6 months in this cohort, perhaps due to heighted risk of morbidity and mortality during the first 3 months of life. Still, EBF to 6 months of age still serves protective purposes during early life in this environment, as evident by the significantly greater number of morbidities experienced at 6 months of age by infants no longer EBF. Feeding practice was also significantly linked to differences in total number of reported infant morbidities in the HERO-G subsample. The greater number of morbidity occurrences reported across the first 12 months of life in infants EBF <6 months may relate to the consequent decrease in immunological protection provided by maternal milk.

Reduced milk consumption during the complementary feeding transition, which decreases the amount of immunological properties consumed by the infant, and introduction of non-breast milk foods, which exposes infants to new non-digestible plant carbohydrates, animal protein, and fats, and provides new substrates for growth of foreign bacterial species, may explain this difference in morbidity occurrence[512–514]. In addition to these exposures, reduction in milk intake by infants also results in a reduction of the protective immunoregulatory components it provides, such as antibodies, oligosaccharides, leukocytes, and cytokines[515]. Moreover, early introduction of complementary foods in resource-limited environments such as in rural Gambia increases the risk of exposure to contaminated foods or water, including that due to pathogens foreign to the immature infant immune system[36163,70,130]. These factors may explain, in part, why the PLS models for "real time" effects of diet on health outcomes at 3 and 6 months of age explained a greater amount of the variation in the dataset relative to the 9 and 12 month time points.

A review of infant morbidity in populations from low-, middle-, and high-income countries found that exclusive breastfeeding to 6 months of age resulted in significantly lower morbidity from GI infection compared to those exclusively breastfed for 3-4 months with continued breastfeeding[516]. Other studies have found that Gambian infants EBF ≥6mo are at lower risk of morbidity, particularly GI infections, which are commonplace in rural, sub-tropical settings (12% prevalence of diarrheal morbidity in urban Gambian infants under 5 years of age)[87,321,517,518]. Birth season, which may influence breastfeeding practices due to increases in maternal agricultural workload, had an important influence on infant morbidity during the first 3 months and at 12 months of life, suggesting persistent effects of the environmental conditions at the time of birth on health outcomes, perhaps through immune programming. Evidence suggests that early life environmental factors, such as season of birth, influence later life health in this

population through epigenetic mechanisms and through the impact of repeated episodes of morbidities on the infant's developing immune system[126].

Milk constituents have numerous immunological roles and their functionality can vary over time. They may also have different functions at different local concentrations. For example, low concentrations of certain peptides are immunomodulatory, whereas higher concentrations are lethal to pathogens. Similarly, some milk constituents may be optimized to inhibit pathogens in the absence of inflammation, whereas others inhibit best in an inflamed environment. Greater relative abundances of undecorated HMO, sia-fuc HMO, and milk IgA were associated with a greater number of infant morbidities.

PLS regressions showed that greater relative abundance of sia-fuc HMO was significantly associated with fewer morbidity occurrences between 6 and 12 months of life. The mixed effects model also showed that greater concentration of sia-fuc HMO significantly predicted fewer morbidity occurrences. Sialic acid has been shown to have immunomodulatory effects and other studies have also connected its abundance to human health outcomes. This suggests that the sialylated HMO structure in particular may have a modulating role in immune defense mechanisms. It has been shown that HMOs need to be both sialylated and fucosylated in order to have impacts on certain components of the immune defense system. For example, sialylation and fucosylation are required to reduce selectin mediated leukocyte rolling, adhesion, and activation, which may protect breastfed infants from excessive immune responses[418,519,520]. Additionally, in non-human models, a single HMO that carries not one but two sialic acids protects infants from necrotizing enterocolitis, one of the most common and fatal GI disorders among preterm infants[418].

While no other HMO classes were significant predictors of infant morbidity in the mixed effects model, relative abundances of fucosylated, sialylated, and undecorated HMO classes all had important associations with infant health outcomes across the first year of life in the PLS regression models. In particular, undecorated HMO showed both real time and extended effects on morbidity occurrence, being positively associated with greater morbidity occurrence, whereas fucosylated HMO had an inverse relationship with morbidity at 3 months of age. Undecorated and fucosylated HMOs have been shown to elicit antimicrobial and anti-inflammatory effects, and are also instrumental in shaping the infant gut microbiota composition. In the gut, these HMOs are primary food substrates for key gut bacteria. Finally, sialylated HMO had an important association with morbidity outcomes at 9 and 12 months of age, which may suggest shifts in immunological needs. This may relate to present or active immunological challenges and/or development stage of infant immune function. Because the relative abundance of undecorated HMO

class was dropped from the mixed effects model, any influence it had on infant health outcomes was missed. By breaking down the analyses into separate time points, individual relationships between morbidity occurrence in early life (and extended into later ages) were more apparent.

Although mixed effects models did not detect a significant association between milk lactose and infant morbidity, PLS regression analyses showed that lactose content had an inverse relationship with infant morbidity occurrence across the first year of life. Lactose can enhance infant nutrition by increasing absorption of minerals including calcium, iron, and zinc[452], which are involved in several immunological defense mechanisms[521–523]. Additionally, lactose is considered an inducer of innate immunity as it upregulates the antimicrobial peptide gene that is protective against pathogens in the gastrointestinal tract[524].

Fat content in maternal milk did not have a strong association with infant morbidity occurrence at any time in the PLS regression analysis. However, the mixed effects model showed that higher milk fat content was a significant predictor of a greater number of morbidity occurrences. Fat is the most highly variable macronutrient in human milk, which may relate to the variation in results between the PLS regressions and mixed effects model. This finding may also relate to the higher fat content in milk collected at 9 and 12 months of lactation relative to that collected at 6 months (see Chapter 3)At 9 and 12 months of lactation, maternal milk fat content was significantly higher than at 6 months of age (see Chapter 3). At 6 months of age, infants are beginning to receive more complementary foods and are thus exposed to new immunological challenges. Other evidence suggests that milk fat would have a protective effect on the infant immune system. Specifically, the structure of milk fat globule may benefit infants by aiding in structural and functional maturation of the gut via the provision of essential nutrients, or may regulate certain cellular events involved in infant growth and immune maturation[525].

Lactoferrin did not have a significant association with morbidity in the PLS regression analyses or mixed effects model. The other HMGP investigated here, IgA, did have positive relationships with morbidity occurrence at 6 and 12 months of age in the PLS regression. The mixed effects model did not find a significant predictive effect of milk IgA on infant health outcomes.

Household SEP score was not influential on infant health outcomes across the first 12 months of life in this analysis. This lack of a relationship suggests that other factors are stronger drivers of infant health, such as infant diet. This has been described in other studies. For example, Gibbs et al. (2014) found that infant feeding practices mediated the role between SEP and early childhood overnutrition[526]. However, others have found the opposite to be true.

Household flooring type, a component of the SEP score calculated here, and source of water have been shown to impact exposures to pathogens and subsequently health outcomes and occurrence of morbidity[527]. With age, behavioral development plays a role in increased exposure to contaminated environments (e.g., infants crawling on the ground), thus material features of the household are in a position to influence infant outcomes[295,361]. The sociodemographic data collected as a part of the larger HERO-G study and variables that are commonly used in SEP calculations are described in more detail in Chapter 2. The household SEP calculation implemented here has potential limitations. This is described in greater detail in the Discussion section of Chapter 2.

Proximity of living area to the MRC clinic in Keneba has been shown to influence health-seeking behavior[505]. This may result from issues such as access to transportation, occupation demands, education level, gender disparities, household income, or perceived nature and/or severity of illness. However, I did not find an impact of village proximity on infant morbidity occurrence in the mixed effects model, and no significant association was observed between distance to MRC clinic and total morbidity occurrence at 3,6, or 9 months of age.

Using morbidity diagnoses collected at clinic visits, this study used the presence or absence of symptoms as an indicator of immune activation. This approach allows for an investigation of specific morbidity types in relationship to early life diet. Additionally, using cumulative morbidity occurrence as the output variable allows for investigation of the role of diet on recurrent illness burdens, with frequent recurrence potentially driving other developmental outcomes in early life. It is important to note that symptomatic morbidity occurrence is only a rough proxy of immune activation because immunological defense mechanisms can still be active even if an individual is asymptomatic. Though beyond the scope of the present study, future work could incorporate an investigation of inflammatory markers (such as CRP) and assess any relationships between immune activation and specific components of maternal milk and EBF duration.

CONCLUSION

The goal of Chapter 4 was to present an overview of research on infant morbidity in The Gambia in relationship to early life diet, and conduct my own assessment of infant morbidity occurrence in relationship to maternal milk composition and complementary feeding practices across the first 12 months of life in the HERO-G subsample. Through use of the dietary questionnaire data presented in Chapter 2 and infant morbidity occurrence data recorded by clinicians at the MRC Keneba field station at clinic visits, I found that infants exclusively breastfed to 6

months or longer had significantly fewer morbidity reports relative to those who began receiving non-breast milk foods earlier. Mixed model results showed that longer exclusive breastfeeding duration predicted significantly fewer occurrences of infant morbidity across the first year of life, and PLS models show both "real time" and "extended" effects of exclusive breastfeeding duration on infant morbidity occurrence. This suggests that illness during infancy and childhood in this environment may be mediated by factors related to breastfeeding, such as milk composition and timing of introduction of non-breast milk foods. All of the milk constituents quantified here (see Chapter 3) were influential on infant morbidity occurrence except fat and lactoferrin. Greater total milk protein content and relative abundance of sialylated HMO predicted a greater number of morbidity occurrences, and higher concentrations of TRP predicted lower morbidity across the first year of life. True protein includes immune proteins, which have established protective effects for infant immune system maturation. Similarly to this, sialylated and undecorated HMO structures illicit protective effects and are equipped to treat features of chronic inflammation. The positive association between the latter milk constituents and infant morbidity may reflect maternal milk synthesis responding to infant needs. The negative relationships between morbidity and abundance of fucosylated and sia-fuc HMOs aligns with research from other studies. Future research incorporating a detailed investigation of the milk proteome and individual HMO structures in relationship to infant outcomes in this population would supplement the present analyses.

This work contributes to our understanding of the interconnections between first foods and infant morbidity. Infants must make a myriad of physiological adjustments during early life. During this critical developmental period, challenges such as undernutrition or chronic infection are drivers of adaptive expansion of the immune system. Such challenges may negatively impact growth outcomes and/or have permanent effects on other aspects of longer-term health outcomes. Detailed longitudinal infant morbidity data from a rural Gambian population allows for a strong model for assessing the influence of early life conditions on health-related outcomes under strong environmental stressors. A deeper understanding of health status during early life in this population helps improve our interpretations and investigations of the complex undernutrition-infection cycle during early life, and will allow for more thorough investigations into the lasting impacts on offspring development.

In Chapters 2-4, I used existing datasets and analyzed biological samples from a subsample of mother-infant pairs in the HERO-G cohort to: (1) characterize breastfeeding and complementary feeding practices in the population; (2) assess maternal milk nutritional and bioactive composition across the first 12 months of lactation; and (3) describe patterns of infant morbidity occurrence over the first year of life. These analyses were contextualized and presented

in relationship to existing literature. From here, I will synthesize and use the data from Chapter 2-4 to inform and justify the analyses in Chapter 5, where I investigate the impact of first foods (exclusive breastfeeding duration and maternal milk composition) and early life health (morbidity occurrence) on infant growth outcomes in this rural Gambian environment.

CHAPTER 5. RELATIONSHIPS BETWEEN MATERNAL MILK COMPOSITION, EXCLUSIVE BREASTFEEDING DURATION, INFANT HEALTH & GROWTH

INTRODUCTION

Infancy and childhood are periods of rapid growth, which requires considerable energetic investment by offspring. Activating immunological defenses in response to illness or infection also carries bioenergetic and metabolic costs[528]. Chronic inflammation or repeated morbidities can lead to poor nutritional status, and undernutrition can increase risk of future infections. In early life, the cumulative impact of infections – especially those of respiratory and gastrointestinal origin – have been shown to impact linear growth outcomes, in particular increasing risk of growth stunting[529,530]. Breastfed infants have different growth patterns compared to non-breastfed infants[531] and maternal milk composition has well-established benefits on health and growth outcomes. Some of these effects relate to interactions between certain components of maternal milk and infant gut microbiota. Adaptation of the mucosal immune system, including functional adaptations of gut microbiota, in response to local disease ecology and environmental stressors is a critical process during early life. It also has implications for longer term outcomes. Although breastfeeding rates are high, morbidity and growth faltering are commonly observed in infancy and early childhood in many low income populations in sub-Saharan Africa, including The Gambia[235]. Longitudinal investigations of the nuanced contribution of breastfeeding practices and an array of nutritional and bioactive components in mother's milk on infant outcomes in these settings may help identify factors that contribute to patterns of infection and suboptimal growth.

In this Chapter, I investigate how breastfeeding practices and maternal milk composition impact infant health (defined by morbidity occurrence), growth outcomes (WHZ, HAZ, WAZ), and intestinal ecology as defined by gut pH, in a rural Gambian population over the first 12 months of life. These analyses incorporate consideration of maternal milk composition, exclusive breastfeeding duration, environmental factors, sex differences, maternal nutritional status during pregnancy, and demographic characteristics. As an exploratory analysis to investigate possible extended effects of early life diet and health on growth outcomes, anthropometric measurements collected at 9 and 12 months of age were assessed in relationship to exclusive breastfeeding status and maternal milk composition at 3 and 6 months of age.

BACKGROUND

Nutrition and growth

Developing infant physiology is sensitive to nutritional cues, distinguishing the early postnatal period as a window of significant expression of adaptive plasticity[89,532–536]. Continued breastfeeding during the weaning process allows for transfer of nutritional and bioactive factors to offspring, including macronutrients, human milk oligosaccharides (HMOs), and human milk glycoproteins (HMGPs), which can mitigate infection and inflammation as well as promote general health, growth, and development[537,538].

Linear growth faltering, which can lead to stunting, is a proxy of chronic undernutrition. Stunting, as defined by the WHO 2006 Child Growth Standards, includes infants and children who are < -2 SD below the recommended length/height-for-age (HAZ) growth scores. The causes of growth faltering are multidimensional; it often occurs during early infancy, and is linked to numerous causal factors, including maternal nutritional status, diet, infection, socioeconomic position, and environment[112,539–541]. Wasted status, or low weight-for-height (WHZ < -2 SD), is a strong predictor of mortality among children under five[542]. Wasting is a symptom of acute undernutrition, commonly a result of insufficient dietary intake or high morbidity incidence, especially diarrhea, and can impair immune system function. Thus, if infants and children become wasted, they experience rapid weight loss, often a direct result of a combination of infection and undernutrition. Compared to stunting, wasting is a relatively short-term condition and appears to be reversible in nature if the child has an adequate diet and is protected from infections. However, repeated events of wasting in early life may lead to stunting in the long term and increase risk of mortality[530,543].

An estimated 149 million children under the age of five are stunted and nearly 50 million are threatened by wasting[471]. In many low-income countries, growth faltering is common and begins with WHZ and weight-for-age (WAZ) declining from around 3 to 12 months of age[460]. Growth faltering is tightly linked with enteropathy, or chronic inflammation of the mucosa of the small intestine, which is the main site of digestion and nutrient absorption throughout the gastrointestinal tract[61]. In addition, chronic inflammation can lead to poor immunological defense to diarrheal diseases, which are prevalent health issues in environments such as The Gambia[69,481,544]. In older studies, up to 25% of growth faltering in Gambian infants can be explained by the decreased ability to digest lactose, which is associated with mucosal enteropathy in the small intestine[480,481]. Environmental factors such as seasonality and disease load likely contribute to growth stunting as well. Small for gestational age infants are frequently born at the end of the

wet season in The Gambia, which is likely attributable to maternal nutritional stress and disease burden, along with higher physical workloads as mothers are heavily involved in agropastoral subsistence work[216,280,490–493].

Unhygienically prepared complementary foods are channels through which gastrointestinal enteropathy can originate. Upon onset, it becomes a self-perpetuating cycle[61,69,481]. Improving nutritional status during early life (be it through increasing availability of critical nutrients, increased appetite, or favoring the survival and proliferation of certain gut microbiota that support immune defense through anti-inflammatory mechanisms), can reduce or eliminate certain negative impacts of morbidity on growth[75,191,502,540,545–550].

Maternal milk can communicate with infant cellular systems and contribute protective effects to the infant through the gut. A healthy gastrointestinal is a physiologic barrier to prevent bacteria and endotoxins from reaching circulation, and there is evidence to suggest that if the intestinal barrier is impaired, it can negatively impact health and growth outcomes. In The Gambia, persistent and chronic damage to the infant gut has caused increasing intestinal permeability, both of which are associated with poor growth outcomes[61,69,478,480]. Specifically, up to 64% of observed height and weight faltering of rural Gambian infants could be explained by increased intestinal permeability via impaired small intestinal mucosal function in older studies from the West Kiang region[69,481]. Although exact causes of the enteropathy are unknown, the intestinal damage documented in this population begins around 6 months of age, coinciding with the timing of introduction of home-prepared complementary foods.

A healthy gut has been linked with reduced risk of other morbidities, such as respiratory and dermatological infections. Inflammatory conditions in the gut are linked to inflammation at other body sites, such as the lungs, with a connection between gut dysbiosis and asthma and chronic obstructive pulmonary disease[551,552]. Intestinal inflammation and gut dysbiosis are also associated with an increased risk of developing eczema during early childhood; however, the role of the gut microbiome in the onset and severity of atopic dermatitis remains debated[553,554].

Co-evolution of milk and infant gut microbiota (*Bifidobacteria*)

Maternal milk is equipped to nourish offspring, and also serves as the sole food source for certain infant gut microbes[465]. The beneficial gut microbe *Bifidobacterium* is adapted to selectively consume human milk oligosaccharides (HMOs) and certain human milk glycoproteins (HMGPs)[203–206]. In particular, *Bifidobacterium longum* subsp. *infantis (B. infantis)* utilizes fucosylated HMOs such as 2'-Fucosyllactose (2'FL). This metabolic process results in organic acid byproducts, which lower GI pH. A lower pH, or more acidic, gut environment is

inhospitable for many pathogenic bacteria, and at the same time fosters further growth of beneficial bifidobacteria[175,207]. Elevated infant fecal pH can indicate a reduction in bifidobacteria abundance[208,209], and dysbiosis of gut microbiota, which is in turn linked to chronic inflammation and GI morbidity[555,556]. Repeated incidences of GI morbidity during early life, particularly common among weaning infants in low-income populations, can lead to further dysbiosis and contribute to increased risk of infection and growth faltering[557].

Growth faltering episodes commonly begin in the first 6 months of life in rural Gambian infants, as does prevalence of intestinal diseases[69,264,307]. Diarrhea was reported as the leading disease contributing to weight faltering in rural Gambian infants, followed by respiratory infection[482]. Research has demonstrated that breastfeeding can reduce risk of diarrheal morbidities in infants[87]. In the West Kiang region of The Gambia, diarrhea in weaning infants was four times more frequent compared to those exclusively breastfed[518]. However, diarrhea had no significant impact on growth for exclusively breastfed infants, suggesting that breastfeeding may buffer weight loss or compromised growth caused by diarrhea in infants[482]. In a recent study, exclusive breastfeeding to 6 months of age showed limited benefit to rural Gambian infant growth outcomes[264]. These results align with other findings from other studies of early life feeding practices and infant growth in low- and middle- income countries (LMICs)[495,558–565]. Alternative explanations provided for the weak associations between infant feeding practice and infant growth in these cohorts include high mean exclusive breastfeeding duration (e.g., >5 but <6 months of age) and strong environmental factors, such as high infectious disease load, poor sanitation, and food insecurity, which may reduce the visibility of the extent of impact of exclusive breastfeeding duration. In Chapter 4, results from PLS regression analyses showed that exclusive breastfeeding duration was a significant predictor of infant morbidity. At 3 months of age, infants EBF for <3 months had a greater number of cumulative morbidity occurrences compared to those EBF ≥3 months. At 6 months of age, infants EBF for <6mo were associated with a greater number of morbidity occurrences relative to those exclusively breastfed ≥6mo.

Milk and gut microbes

Despite the low pH throughout the digestive process, HMOs and HMGPs can survive the acidic conditions and can reach the colon, where they serve as substrate for the developing infant gut microbiota, fostering growth of key gut microbes such as *Bifidobacterium*[191,566,567]. In breastfed human infants, the beneficial bifidobacteria represent

up to 90% of gut microbiota[568–570]. Similar to functions of HMOs and HMGPs, bifidobacteria aid in infant immune system development, decrease inflammation, help prevent infection, and can treat GI morbidities[498,554].

Growing evidence demonstrates that the interrelationships between HMOs, HMGPs, and bifidobacteria can influence offspring health, growth, and survival. As such, these milk constituents and their interplay with certain infant gut microbes can contribute to maternal future reproductive success and fitness. Around 1% of HMO content is absorbed into the infant bloodstream while the remaining ~99% are consumed by intestinal microbes or excreted in feces or urine[344]. This suggests that an infant with certain gut microbiota profiles – particularly those dominated by *Bifidobacterium* – may be capable of optimizing maternal milk bioactives for improved health and growth. These relationships may be particularly important in low-income populations in environments with high pathogen load and food insecurity.

Fecal pH

The acidity or alkalinity of the gut environment, as defined by pH, can greatly affect microbial survival and proliferation[571]. The interaction between HMOs, HMGPs, and lactic acid bacteria (e.g., *Bifidobacterium* and *Lactobacillus*) results in a primary fermentative output of organic acids such as acetate and lactate, thereby creating a more acidic gut environment[203–206]. Lower gut pH subsequently prevents colonization by pathogenic bacteria and creates a more hospitable landscape for the beneficial bifidobacteria, providing numerous health benefits to offspring[207]. Thus, interactions between human milk bioactives and certain gut bacteria – particularly bifidobacteria – can optimize the intestinal phenotype to match stressors such as vulnerability to infection[175,207].

Loss or absence of bifidobacteria in the infant gut microbiome is marked by loss or significant decrease of this organic acid production, which results in elevated fecal pH (flagged by pH > 5.5)[208,209]. Because of its correlation with bifidobacteria abundance, increased intestinal pH may indicate disruption of the infant gut microbiota, which may lead to chronic inflammation or immune-mediated diseases[555,556]. These perturbations may have long-lasting effects. Additionally, a significant inverse association between fecal pH and child growth was recently reported in a population of rural Bangladeshi infants, reflecting the range of health indicators that fecal pH may represent[572]. As such, maintaining certain conditions within the infant gut suited to support survival and proliferation of *Bifidobacterium* is advantageous. Moreover, this evidence suggests that selective pressures on lactation and milk bioactive composition were driven, at least in part, by gut microbiota. Fecal pH and its association with infant gut

microbial composition in infants from diverse populations who are exclusively breast fed and those who are receiving complementary foods has not been comprehensively investigated, particularly among those with heavy disease load.

Infants receiving complementary food experienced diarrhea four times more frequently than exclusively breastfed infants in rural Gambia[518]. Diarrheal disease becomes more prevalent around 3 to 6 months of age in rural Gambia, coinciding with the average weaning age[307]. Growth stunting parallels the onset of diarrheal disease during this time. Numerous pathologies were discovered in small bowel biopsies of Gambian children, including flattened villi and elevated intraepithelial CD8 lymphocytes, which are suggestive of pro-inflammatory GI states[78,479,573]. Recent research shows that rural Gambian infant GI tracts are dominated by the beneficial bifidobacteria, representing around 70% of total bacteria across multiple time points within the first 6 months of life[574]. In the same study, when bifidobacteria levels decreased significantly, there were concomitant increases in infant morbidity and/or growth faltering, as well as large shifts in milk HMO profiles. Thus, gut microbiota appear to have associations with HMO composition, infant morbidity, and infant growth outcomes in this region.

Here, longitudinal data on infant feeding practices, maternal milk composition, infant morbidity occurrence, fecal pH (as a proxy for intestinal ecology), and anthropometric measures over the first 12 months of life were used to investigate the influence of EBF duration and morbidity incidence on rural Gambian infant growth outcomes.

Maternal nutrition (MUAC) during pregnancy and infant growth outcomes

Several studies have found that maternal nutrition during conception and throughout gestation are associated with child anthropometrics[575,576]. Low body mass and short stature, which are both prevalent in low income countries, can lead to poor fetal development and higher risk of complications in pregnancy[125]. In The Gambia, chronically undernourished women given prenatal dietary supplementation showed reduced retardation in intrauterine growth of their infants[577]. It was also associated with a significant reduction in prevalence of stillbirth and early neonatal mortality. Another Gambian study found that greater weight gain during the second and third trimester (beyond a certain threshold) may be protective against small for gestational age[578].

RESEARCH AIMS

Aims

In this Chapter, I combine the analyses conducted in Chapters 2-4 and assess associations between exclusive breastfeeding duration, nutritional and bioactive composition of maternal milk, and infant health (as defined by documented morbidity occurrence) and their influence on infant growth outcomes during the first year of life in a rural Gambian population. To investigate possible extended effects of early life diet and morbidity occurrence on growth outcomes, growth outcomes at 9 and 12 months of age are also assessed in relationship to exclusive breastfeeding status, maternal milk composition, and cumulative morbidity occurrence at 3 and 6 months of age. Statistical models will include consideration of sex differences, maternal factors, environmental conditions, and socioeconomic characteristics. Additionally, I present results from an exploratory analysis of infant intestinal ecology (as represented by fecal pH) over the first year of life, which provides insight into the landscape of the body site where first foods interact with gut microbiota known to influence infant health and growth outcomes.

I will answer the following research question:

What are the effects of early life diet (including maternal milk composition and infant complementary feeding practices) and infant morbidity on growth during the first year of life?

METHODS

Sample size

The HERO-G subsample (N=194) (inclusion criteria detailed in Chapter 2) was selected for the present analysis of relationships between diet (EBF duration and maternal milk composition), morbidity occurrence, and infant growth outcomes.

Infant dietary questionnaires

Dietary questionnaires regarding infant feeding were administered to mothers by trained field workers every 10 days starting at one week of infant age until 12 months of infant age. Infant feeding practice was defined by exclusive breastfeeding status at 6 months of age, based on the WHO recommended exclusive breastfeeding duration of 6 months of age[321]. Infants were categorized as either ‘EBF <6mo’ (provision of breast milk and non-breast milk

foods/liquids before 6mo) or 'EBF ≥6mo' (provision of breast milk only until 6 months or later)[264,322]. Additional details regarding infant dietary questionnaire methodology can be found in Chapter 2.

Maternal milk composition

Concentrations of maternal milk macronutrients (fat, protein, lactose, true protein) and the relative abundances of human milk oligosaccharide classes (fucosylated, sialylated, undecorated, sialylated and fucosylated [sia-fuc]) and human milk glycoproteins (lactoferrin, IgA) were analyzed in a subset of 50 mothers in the HERO-G cohort at 3, 6, 9, and 12 months of lactation following the methodology described in Chapter 3. Briefly, mid-infrared technology was used to measure the milk macronutrient concentrations, and mass spectrometry was implemented to quantify the relative abundances of the HMO classes and the HMGPs in samples collected a subset of the HERO-G subsample across the first year of lactation.

Infant morbidity data

Infant morbidities were diagnosed and recorded by clinicians at the MRC Keneba field station at scheduled clinic visits at 3, 6, 9, 12 months of age, plus non-scheduled visits (caregivers sought and were provided MRC clinical evaluation and treatment for infant morbidity as needed). Additional methodological details are described in Chapter 4.

Anthropometric measurements

Infant

Following their naming ceremony at 1 week of age, anthropometric measurements were collected in triplicate every other day home visits by trained field workers until 12 months of age and at clinic visits at 3, 6, 9, and 12 months of age. Infants were undressed before weights were measured using electronic scales to the nearest 10g (Seca 336 digital weighing scale) and length was measured using length boards (Seca 417) to a precision of 0.1cm. Scales were calibrated each day prior to measurement. Infant growth measurements collected at clinic visits at 3, 6, 9, 12, and 24 months of age were indexed using height-for-age (HAZ), weight-for-age (WAZ), and weight-for-height (WHZ) Z-scores according to the WHO Child Growth Standards by using WHO Anthro program (Version 3.2.2)[84]. Following WHO values, wasted, stunted, and underweight are defined as WHZ, HAZ, and WAZ Z-scores < -2SD, respectively.

This dissertation focuses on growth measurements collected during the first year of life, with a specific focus on the 3, 6, 9, and 12 month collection time points.

Maternal

At a scheduled prenatal clinic visit, a HERO-G study midwife measured maternal mid-upper arm circumference (MUAC) at 36 weeks of gestation, during the third trimester of pregnancy. MUAC was measured using flexible measuring tape (Seca 212) to the nearest 0.1mm[263]. Undernutrition was classified as a MUAC value of < 23 cm[265]. Full methodology is described in Chapter 3.

Secretor status

Secretor status ('Secretor' vs 'non-Secretor') was designated based on relative abundance of known α1–2-linked fucosylated HMOs, including 2′FL, LDFT, TFLNH, DFLNHa, DFLNHc, and IFLNH I. Milk containing >6% relative abundance of α1–2-linked fucosylated HMOs were categorized as phenotypic Secretors[419]. Additional details on this methodology are described in Chapter 3.

Socioeconomic position

Fieldworkers administered a socioeconomic questionnaire during the booking visit for the HERO-G study. Mothers were asked to provide information regarding sociodemographic variables (maternal education attainment), household characteristics (crowding index [number of persons per room within a dwelling], material of dwelling walls and floor), and durable assets (livestock ownership, possession of a cart). Questionnaire responses describing sociodemographic characteristics, household characteristics, and durable assets were used to generate an asset score using Principal Component Analysis (PCA). Complete details on the socioeconomic position (SEP) calculation and categorization are provided in Chapter 2.

Stool sample collection

Prior to each scheduled clinic visit, mothers used a stool collection kit, validated in a prior study[437], to collect a sample of infant stool. Stool samples were stored on ice in a cooler box delivered to the mother's home the day of collection and brought with them to their infant's clinic visits. Samples were processed in the MRC laboratory, and

subsequently kept frozen at -70°C through shipment to the University of Colorado Boulder and onward to the University of California Davis.

Fecal pH protocol

The pH of 450 infant fecal samples collected at 3 (N=107), 6 (N=88), 9 (N=104), and 12 (N=92) months of age were measured using the Orion VersaStar Pro (Thermo Scientific), which has a pH precision of 0.01. First, using a sterile metal spatula, 30-50mg of frozen fecal sample (weighed using Mettler Toledo Analytical Scale) was transferred into a sterile Eppendorf test tube. Sterile distilled water (room temperature) was added to the sample at a 1:9 (wt:vol) ratio following published protocols[579,580]. Diluted fecal samples were then vortexed for 4 minutes Fisher Vortex Genie2 and then centrifuged at 10,000g for 5 minutes. Without making contact with the precipitate, the pH of the sample supernatant was measured using the Orion VersaStar Pro. Samples were stored on dry ice during the preparation phase, and stored for no more than 20 minutes.

Population comparison of infant fecal pH in the last century

Data were compiled from available publications on breastfed infant fecal pH published within the last century in order to compare to the results of our measures of intestinal acidity in the HERO-G cohort. A total of 19 publications were included in the present analysis and were selected based on evaluation in metaanalysis by Henrick et al. (2019).

Statistical analysis

Descriptive statistics were calculated for infant growth outcomes (WHZ, HAZ, WAZ) and infant fecal pH across the first 12 months of life. Partial Least Squares (PLS) regression models were constructed to examine "real time" effects of dietary variables (EBF status and maternal milk composition) and morbidity occurrence on WHZ, HAZ, and WAZ at 3, 6, 9, and 12 months of age. Additional PLS regression models were constructed to investigate the potential extended influence of 3 and 6 month dietary variables and morbidity occurrences on 9 and 12 month growth outcomes. Validation Normalization of the observations (values of both X and Y variables) was achieved using mean centering and unit variance scaling. Results sections of PLS regression model results include presentation of: (1) number of PLS regression factors, with factor being defined as the combination of explanatory variables; (2) description of the percentage of variation explained for cumulative Y (% of variation in output variable explained by

the factor) and the percentage of variation explained for cumulative X (% of variation in explanatory variables explained by output variable) (emphasis is placed on the importance of the percentage of variation explained for cumulative Y); (3) assessment of prediction error sum of squares (PRESS) and the Variable Importance of the Projection (VIP) statistic. Additional detail on PLS regression methodology can be found in the Methods section of Chapter 4. Additionally, mixed effects models were constructed as a comparison of statistical approaches for assessment of potential predictors of WHZ, HAZ, and WAZ during the entire first year of life. Significant associations identified in the mixed effects models were summarized using the beta regression coefficients (B), 95% confidence intervals (CI), and P-values. The level of statistical significance was set to $P < 0.05$ for all analyses. All statistical analyses were conducted using JMP Pro 15.0 statistical software (© 2019 SAS Institute, Inc.). Statistical significance was set at $P<0.05$. Mixed effects model variables and PLS regression model variables at 3, 6, 9, and 12 months of age and the regressions assessing 3 and 6 month dietary variables on 9 and 12 month growth outcomes and infant fecal pH are defined in **Table 5.1-Table 5.9.**

Table 5.1. PLS regression model variables ("Real time" effects: 3 months of age)

Outcome Variables (3mo)	Explanatory Variables
WHZ	• Milk composition at 3mo (Macronutrients, HMOs, HMGPs) • Cumulative morbidity at 3mo (continuous) • EBF status at 3mo (Yes/No) • Maternal Secretor status (Secretor/Non-Secretor) • Maternal MUAC[a] (continuous) • Infant birth wt (continuous) • Infant season of birth (Wet/Dry) • Infant sex (F/M) • Socioeconomic position (SEP score) • Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.2. PLS regression model variables ("Real time" effects: 6 months of age)

Outcome Variables (6mo)	Explanatory Variables
WHZ	• Milk composition at 6mo (Macronutrients, HMOs, HMGPs) • Cumulative morbidity at 6mo (continuous) • EBF status at 6mo (Yes/No) • Maternal Secretor status (Secretor/Non-Secretor) • Maternal MUAC[a] (continuous) • Infant birth wt (continuous) • Infant season of birth (Wet/Dry) • Infant sex (F/M) • Socioeconomic position (SEP score) • Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.3. PLS regression model variables ("Real time" effects: 9mo of age)

Outcome Variables (9mo)	Explanatory Variables
WHZ	• Milk composition at 9mo (Macronutrients, HMOs, HMGPs) • Cumulative morbidity at 9mo (continuous) • Maternal Secretor status (Secretor/Non-Secretor) • Maternal MUAC[a] (continuous) • Infant birth wt (continuous) • Infant season of birth (Wet/Dry) • Infant sex (F/M) • Socioeconomic position (SEP score) • Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.4. PLS regression model variables ("Extended" effects: 9 month outcomes with 3 month dietary and morbidity variables)

Outcome Variables (9mo)	Explanatory Variables
WHZ	• Milk composition at 3mo (Macronutrients, HMOs, HMGPs) • Cumulative morbidity at 3mo (continuous) • EBF status at 3mo (Yes/No) • Maternal Secretor status (Secretor/Non-Secretor) • Maternal MUAC[a] (continuous) • Infant birth wt (continuous) • Infant season of birth (Wet/Dry) • Infant sex (F/M) • Socioeconomic position (SEP score) • Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.5. PLS regression model variables ("Extended" effects: 9 month outcomes with 6 month dietary and morbidity variables)

Outcome Variables (9mo)	Explanatory Variables
WHZ	• Milk composition at 6mo (Macronutrients, HMOs, HMGPs) • Cumulative morbidity at 6mo (continuous) • EBF status at 6mo (Yes/No) • Maternal Secretor status (Secretor/Non-Secretor) • Maternal MUAC[a] (continuous) • Infant birth wt (continuous) • Infant season of birth (Wet/Dry) • Infant sex (F/M) • Socioeconomic position (SEP score) • Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.6. PLS regression model variables ("Real time" effects: 12mo of age)

Outcome Variables (12mo)	Explanatory Variables
WHZ	• Milk composition at 12mo (Macronutrients, HMOs, HMGPs) • Cumulative morbidity at 12mo (continuous) • Maternal Secretor status (Secretor/Non-Secretor) • Maternal MUAC[a] (continuous) • Infant birth wt (continuous) • Infant season of birth (Wet/Dry) • Infant sex (F/M) • Socioeconomic position (SEP score) • Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.7. PLS regression model variables ("Extended" effects: 12 month outcomes with 3 month dietary and morbidity variables)

Outcome Variables (12mo)	Explanatory Variables
WHZ	● Milk composition at 3mo (Macronutrients, HMOs, HMGPs) ● Cumulative morbidity at 3mo (continuous) ● EBF status at 3mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Infant birth wt (continuous) ● Infant season of birth (Wet/Dry) ● Infant sex (F/M) ● Socioeconomic position (SEP score) ● Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.8. PLS regression model variables ("Extended" effects: 12 month outcomes with 6 month dietary and morbidity variables)

Outcome Variables (12mo)	Explanatory Variables
WHZ	● Milk composition at 6mo (Macronutrients, HMOs, HMGPs) ● Cumulative morbidity at 6mo (continuous) ● EBF status at 6mo (Yes/No) ● Maternal Secretor status (Secretor/Non-Secretor) ● Maternal MUAC[a] (continuous) ● Infant birth wt (continuous) ● Infant season of birth (Wet/Dry) ● Infant sex (F/M) ● Socioeconomic position (SEP score) ● Parity (continuous)
HAZ	
WAZ	
*Fecal pH**	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy*; *Note:* Cumulative morbidity was not included in PLS regressions assessing potential predictors of infant fecal pH as it was not expected to be an explanatory variable

Table 5.9. Response and explanatory variables investigated in separate mixed effects model for WHZ, HAZ, and WAZ across the first 12 months of life

Outcome variable*	**Explanatory variable**
WHZ	*Fixed* ● Maternal MUAC[a] (continuous) ● Maternal Secretor status (Secretor/Non-Secretor) ● Infant sex (F/M) ● Infant season of birth (Wet/Dry) ● Socioeconomic position (SEP score) ● Parity (continuous) ● EBF duration (months) ● Collection time point (3, 6, 9, 12mo) ● Milk composition (Macronutrients, HMOs, HMGPs) *Random* ● Subject ID
HAZ	
WAZ	

[a]Maternal MUAC measurements were collected during the third trimester of pregnancy

RESULTS

Maternal/infant characteristics and infant feeding

The baseline characteristics of these subgroups, along with the larger HERO-G cohort, can be found in Chapter 1 and 3. Average infant age at cessation of exclusive breastfeeding in the HERO-G subsample was 5.0 (±1.5) months, with 135 (69.6%) of infants EBF for less than the WHO recommended 6 months and 59 (30.4%) infants EBF ≥6mo. Full details on infant feeding characteristics can be found in Chapter 2.

Growth outcomes across the first 12 months of life

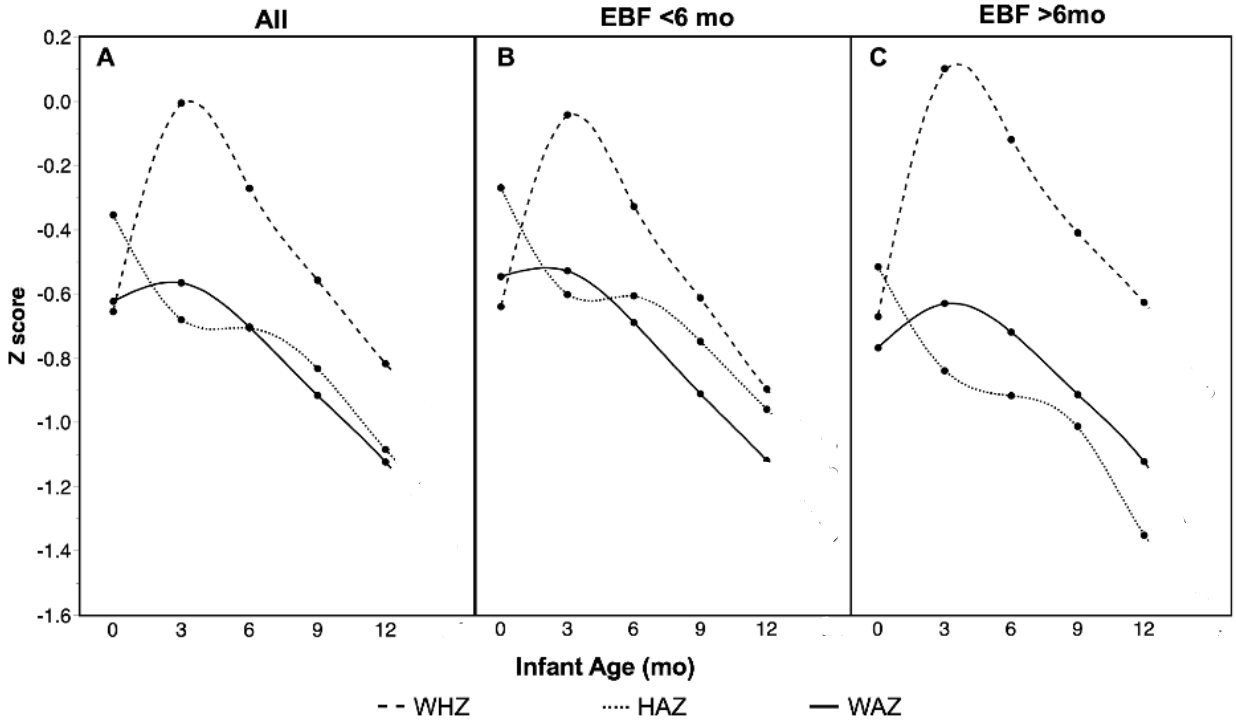
Infant growth outcomes at 3 (N=185), 6 (N=184), 9 (N=186) and 12 (N=190) months were assessed. At 3 months of age, 6 (3.2%) of the HERO-G subsample infants were classified as wasted (WHZ < -2 SD), 13 (7.1%) as stunted (HAZ < -2 SD), and 15 (8.1%) as underweight (WAZ < -2 SD). All others were within the WHO defined "normal" Z-score range. At 6 months of age, 13 (5.9%) were classified as wasted, 19 (10.3%) as stunted, and 12 (8.1%) as underweight. At 9 months of age, 14 (7.5%) of infants were classified as wasted, 21 (11.3%) as stunted, and 22 (11.8%) as underweight. By 12 months of age, these numbers continued to climb: 25 (13.2%) were classified as wasted, 32 (16.9%) as stunted, and 34 (17.9%) as underweight. Wasted, stunted, and underweight distributions at 3, 6, 9, and 12 months of age are detailed in **Table 5.10**. Infant growth trajectories across the first 12 months of life are illustrated in **Figure 5.1.**

Table 5.10. Distribution of wasted, stunted, and/or underweight infants at 3, 6, 9, and 12 months of age

	Age (mo)	**3 (N=185)**	**6 (N=184)***	**9 (N=186)**	**12 (N=190)**
WHZ	Wasted	6 (3.2)	13 (5.9)	14 (7.5)	25 (13.2)
	Normal	179 (96.8)	173 (94.0)	172 (92.5)	165 (86.8)
HAZ	Stunted	13 (7.1)	19 (10.3)	21 (11.3)	32 (16.9)
	Normal	171 (92.9)	165 (89.7)	165 (88.7)	158 (83.2)
WAZ	Underweight	15 (8.1)	12 (8.1)	22 (11.8)	34 (17.9)
	Normal	170 (91.9)	170 (91.9)	164 (88.2)	156 (82.1)

Data are presented as N (%); *6mo: WHZ & HAZ N=184; WAZ N=185; *24mo WHZ N=180; HAZ & WAZ N=181

Figure 5.1. Infant WHZ, HAZ, and WAZ growth trajectories across the first 12 months of life in (A) the HERO-G subsample; (B) infants EBF <6mo (N=135); and infants EBF ≥6mo (N=59)



PLS regression model results (predictors of infant growth outcomes)

WHZ

Infant WHZ across the first year of life was assessed in relationship to infant dietary, maternal, environmental, and sociodemographic factors using PLS regression models. Results are described below according to time point and detailed in **Table 5.11** through **Table 5.13**.

WHZ at 3 months of age

In the PLS regression assessing potential drivers of WHZ at the 3 month time point, the minimum root PRESS, the measure of the predictive power of the model, was 0.9952, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 31.1% and the percent variation explained for cumulative Y was 62.1%. The 1-component model showed 9 influential variables (VIP > 1.0) on infant WHZ at 3 months of age.

Milk composition at 3 months of lactation and EBF status had important influence on WHZ at 3 months of age. Lower WHZ was predicted by higher concentrations of protein (VIP: 1.5021; B: -0.0939 WHZs), TRP (VIP:

1.0625; B: -0.0664 WHZs), and relative abundances of fucosylated HMO (VIP: 1.0764; B: -0.0673 WHZs) and sia-fuc HMO (VIP: 1.0350; B: -0.0647 WHZs), whereas greater relative abundance of undecorated HMO predicted higher WHZ (VIP: 1.0284; B: 0.0643). EBF to 3 months of age predicted lower WHZ by -0.0627 WHZs (VIP: 1.0029) compared to infants no longer EBF at 3 months. In addition to these dietary variables, maternal and environmental factors also influenced WHZ at 3 months of age. Maternal parity had the strongest influence on WHZ at 3 months of age, with greater parity predicting lower WHZ (VIP: 2.3098; B: -0.1444). Being born during the dry season predicted higher WHZ (VIP: 1.6079; B: 0.1005) compared to being born in the wet season.

WHZ at 6 months of age

In the PLS regression assessing potential drivers of WHZ at the 6 month time point, the minimum root PRESS was 0.9828, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 47.2% and the percent variation explained for cumulative Y was 64.6%. The 1-component model showed 7 influential variables (VIP > 1.0) on infant WHZ at 6 months of age.

Lower WHZ at 6 months of age was predicted by greater relative abundances of sia-fuc HMO (VIP: 1.4948; B: -0.00972 WHZs) and IgA (VIP: 1.2225; B -0.0795 WHZs) collected at 6 months of lactation. EBF status at 6 months had a strong influence on 6 month WHZ, with EBF to 6 months of age predicting higher WHZ by 0.1196 WHZs (VIP: 1.9392). In addition to these dietary variables, infant and maternal factors also influenced WHZ at 6 months of age. Birth weight had the strongest influence on WHZ at 6 months of age (VIP: 2.0812; B: 0.1353 WHZs). Infant morbidity occurrence by 6 months negatively influenced WHZ by -0.1085 WHZs (VIP: 1.6689). Higher maternal parity predicted lower WHZ by -0.0926 WHZs (VIP: 1.4240). Maternal Secretor status also had an important influence on WHZ at 6 months, with non-Secretor status predicting lower WHZ by -0.0728 (VIP: 1.1195).

WHZ at 9 months of age

"Real time" effects. In the PLS regression assessing potential drivers of WHZ at the 3 month time point, the minimum root PRESS was 0.9952, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 39.8% and the percent variation explained for cumulative Y was 49.4%. The 1-component model showed 4 influential variables (VIP > 1.0) on infant WHZ by 9 months of age.

Milk protein content at 9 months of lactation was the only influential dietary variable on WHZ at 9 months of age, with greater protein content predicting lower WHZ by -0.1104 WHZs (VIP: 1.6675). Other maternal, infant, and environmental factors also had strong influence on 9 month WHZ. Infant morbidity occurrence had the strongest influence on WHZ at 9 months of age, with a higher number of morbidity occurrences predicting lower WHZ by -0.1491 WHZs (VIP: 2.2512). Birth during the dry season predicted lower WHZ at 9 months by -0.1188 WHZs (VIP: 1.7932). Maternal parity also influenced WHZ at 9 months, with higher parity predicting lower WHZ by -0.1221 WHZs (VIP: 1.8433).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant WHZ at 9 months of age had a minimum root mean PRESS of 0.9882, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 57.8% and 76.4% for the percentage of variation explained for cumulative Y. The 1-component model showed 4 influential variables (VIP > 1.0) on infant WHZ at 9 months of age.

Milk protein content at 3 months of lactation was the only dietary influence of WHZ at 9 months of age, with greater protein content predicting lower WHZ (VIP: 1.7450; B: -0.1105 WHZs). Other maternal, infant, and environmental variables were also influential. Total morbidity at 3 months of age had the strongest influence on WHZ at 9 months of age, with higher number of morbidity occurrences predicting lower WHZ by -0.1492 WHZs (VIP: 2.3560). Birth during the dry season predicted higher WHZ by 0.1188 WHZs (VIP: 1.8766). Finally, higher maternal parity predicted lower WHZ by -0.1221 (VIP: 1.9290).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant WHZ at 9 months of age had a minimum root mean PRESS of 0.9941, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 67.4% and 74.0% for the percentage of variation explained for cumulative Y. The 1-component model showed 5 influential variables (VIP > 1.0) on infant WHZ at 9 months of age.

Milk protein and EBF status at 6 months were the only influential dietary variables on WHZ at 9 months of age. Greater milk protein content predicted lower WHZ by -0.1033 WHZs (VIP: 1.6441), and EBF to 6 months of age predicted higher WHZ by 0.0714 WHZs (VIP:1.1365). In addition to these dietary variables, other maternal, infant,

and environmental variables were influential on WHZ at 9 months. Total morbidity at 6 months of age had the strongest influence on 9 month WHZ, with greater number of morbidities predicting lower WHZ by -0.1394 WHZs (VIP: 2.2197). Maternal parity also had a negative association with 9 month WHZ, with higher parity predicting lower WHZ (VIP: 1.8175; B: -0.1142 WHZs). Finally, birth during the dry season predicted higher WHZ at 9 months by 0.1111 WHZs (VIP: 1.7681) relative to birth during the wet season.

WHZ at 12 months of age

"Real time" effects. In the PLS regression assessing potential drivers of WHZ at the 12 month time point, the minimum root PRESS was 0.9851, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 38.1% and the percent variation explained for cumulative Y was 61.9%. The 1-component model showed 7 influential variables (VIP > 1.0) on infant WHZ at 12 months of age.

Milk composition at 12 months of lactation had strong influence on WHZ at 12 months of age. Greater protein and relative abundance of milk IgA predicted lower WHZ at 12 months by -0.0647 WHZs (VIP: 1.2446) and -0.0594 WHZs (VIP: 1.1437), respectively. Higher WHZ was predicted by greater lactose content in milk (VIP: 1.2014; B: 0.0624). In addition to these dietary variables, other maternal, infant, and environmental variables had strong influence on 12 month WHZ. Total morbidity occurrence had the strongest influence on WHZ at 12 months of age, with greater number of morbidities predicting lower WHZ by -0.1085 WHZs (VIP: 2.0872). Birth during the dry season had a strong negative association with WHZ at 12 months (VIP: 1.6974; B: -0.0882). Maternal nutrition during the 3rd trimester also had a strong negative association with 12 month WHZ, with higher MUAC measurements predicting lower WHZ by -0.0717 WHZs (VIP: 1.3790). Finally, higher parity predicted lower WHZ by -0.0541 WHZs (VIP: 1.0421).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant WHZ at 12 months of age had a minimum root mean PRESS of 0.9574, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 49.0% and 72.0% for the percentage of variation explained for cumulative Y. The 1-component model showed 7 influential variables (VIP > 1.0) on infant WHZ at 12 months of age.

Milk composition at 3 months, but not EBF status, was influential on WHZ at 12 months of age. Lower WHZ at 12 months was predicted by higher 3 month milk protein content by -0.0640 WHZs (VIP: 1.3017) and greater relative abundance of 3 month milk IgA by -0.0588 WHZs (VIP: 1.1962). Higher lactose content in milk collected at 3 months of lactation predicted higher WHZ as 12 months by 0.0618 WHZs (VIP: 1.2565). In addition to these dietary variables, additional maternal, infant, and environmental variables also had strong influence on 12 month WHZ. Morbidity occurrence at 3 months of age had the strongest influence on 12 month WHZ, with a greater number of morbidity occurrences predicting lower WHZ by -0.1073 WHZs (VIP: 2.1830). Birth during the dry season predicted lower WHZ relative to birth during the wet season (VIP: 1.7753; B: -0.0873). Maternal nutritional status during the 3rd trimester also had strong influence on 12 month WHZ; higher maternal MUAC measurements predicted lower WHZ by -0.0709 (VIP: 1.4423). Finally, higher maternal parity predicted lower WHZ at 12 months of age by -0.0536 WHZs (VIP: 1.0899).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant WHZ at 12 months of age had a minimum root mean PRESS of 0.9742, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 54.9% and 82.9% for the percentage of variation explained for cumulative Y. The 1-component model showed that birth season, maternal nutritional status during the 3rd trimester, total infant morbidity occurrence at 6 months of age, EBF status at 6 months, and milk protein and lactose content were influential (VIP > 1.0) on infant WHZ at 12 months of age.

Maternal milk composition and EBF status at 6 months of lactation had significant influences on WHZ at 12 months of age. Lower 12 month WHZ was predicted by higher milk protein content at 6 months of lactation by -0.0635 WHZs (VIP: 1.0782). Lactose content at 6 months of lactation had a positive relationship with WHZ at 12 months of age (VIP: 1.0408; B: 0.0613 WHZs). EBF to 6 months of age had the strongest influence on 12 month WHZ, predicting higher WHZ by 0.1120 WHZs (VIP: 1.9025). In addition to these dietary variables, other maternal, infant, and environmental variables also had strong influence on 12 month WHZ. A greater number of infant morbidity occurrences at 6 months of age predicted lower WHZ at 12 months by -0.1064 WHZs (VIP: 1.8082). Birth during the dry season predicted higher WHZ at 12 months by 0.0865 WHZs (VIP: 1.4705). Higher maternal MUAC during the 3rd trimester had a negative influence on 12 month WHZ, with higher MUAC predicting lower WHZ by -0.0703 WHZs (VIP: 1.1947).

Table 5.11. VIP statistics and model coefficients (B) from PLS regressions assessing "real time" and "extended" predictors of WHZ at 3, 6, 9, and 12 months of age for centered and scaled data

	"Real time" predictors of infant WHZ							
Time Point	**3mo**		**6mo**		**9mo**		**12mo**	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.4904	-0.0307	0.2142	-0.0139	0.1339	0.0089	0.3460	0.0180
Sex[M]	0.4904	0.0307	0.2142	0.0139	0.1339	-0.0089	0.3460	-0.0180
BirthSeason[Dry]	**1.6079**	0.1005	0.0149	-0.001	**1.7932**	0.1188	**1.6974**	0.0882
BirthSeason[Wet]	**1.6079**	-0.1005	0.0149	0.001	**1.7932**	-0.1188	**1.6974**	-0.0882
SEP Score	0.4769	0.0298	0.5399	0.0351	0.0691	0.0046	0.3703	0.0192
Parity	**2.3098**	-0.1444	**1.4240**	-0.0926	**1.8433**	-0.1221	**1.0421**	-0.0541
Maternal MUAC	0.7809	-0.0488	0.2509	-0.0163	0.8956	-0.0593	**1.3790**	-0.0717
SecretorStatus[Non-Secretor]	0.1956	-0.0122	**1.1195**	-0.0728	0.6133	-0.0406	0.6645	-0.0345
SecretorStatus[Secretor]	0.1956	0.0122	**1.1195**	0.0728	0.6133	0.0406	0.6645	0.0345
BirthWt	0.4904	-0.0307	**2.0812**	0.1353	0.1719	0.0114	0.6629	0.0344
TotalMorbidity	0.2075	0.013	**1.6689**	-0.1085	**2.2512**	-0.1491	**2.0872**	-0.1085
EBF Status @3mo [Y]	**1.0029**	-0.0627	-	-	-	-	-	-
EBF Status @3mo [N]	**1.0029**	0.0627	-	-	-	-	-	-
EBF Status @6mo [Y]	-	-	**1.8392**	0.1196	-	-	-	-
EBF Status @6mo[N]	-	-	**1.8392**	-0.1196	-	-	-	-
Fat	0.5727	-0.0358	0.2435	0.0158	0.4239	-0.0281	0.3593	0.0187
Protein	**1.5021**	-0.0939	0.0181	0.0012	**1.6675**	-0.1104	**1.2446**	-0.0647
Lactose	0.8319	0.052	0.0559	0.0036	0.4059	0.0269	**1.2014**	0.0624
True Protein	**1.0625**	-0.0664	0.1187	0.0077	0.7558	-0.0501	0.3854	-0.0200
Fucosylated HMO	**1.0764**	-0.0673	0.1233	-0.008	0.4957	0.0328	0.5786	0.0301
Sialylated HMO	0.8775	0.0549	0.4491	-0.0292	0.2909	-0.0193	0.1535	0.008
Undecorated HMO	**1.0284**	0.0643	0.3021	0.0196	0.4847	-0.0321	0.5828	-0.0303
Sia-fuc HMO	**1.0350**	-0.0647	**1.4948**	-0.0972	0.0969	0.0064	0.7431	-0.0386
Lactoferrin	0.5752	-0.0360	0.1521	-0.0099	0.0024	-0.0002	0.6052	-0.0314
IgA	0.1043	0.0065	**1.2225**	-0.0795	0.4561	-0.0302	**1.1437**	-0.0594
	"Extended" predictors of morbidity occurrence							
Time Point	**9mo_3mo**		**9mo_6mo**		**12mo_3mo**		**12mo_6mo**	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.1402	0.0089	0.1321	0.0083	0.3619	0.0178	0.2998	0.0176
Sex[M]	0.1402	-0.0089	0.1321	-0.0083	0.3619	-0.0178	0.2998	-0.0176
BirthSeason[Dry]	**1.8766**	0.1188	**1.7681**	0.1111	**1.7753**	0.0873	**1.4705**	0.0865
BirthSeason[Wet]	**1.8766**	-0.1188	**1.7681**	-0.1111	**1.7753**	-0.0873	**1.4705**	-0.0865
SEP Score	0.0723	0.0046	0.0681	0.0043	0.3873	0.019	0.3208	0.0189
Parity	**1.9290**	-0.1221	**1.8175**	-0.1142	**1.0899**	-0.0536	0.9028	-0.0531
Maternal MUAC	0.9373	-0.0593	0.8831	-0.0555	**1.4423**	-0.0709	**1.1947**	-0.0703
SecretorStatus[Non-Secretor]	0.6418	-0.0406	0.6047	-0.0380	0.6950	-0.0342	0.5756	-0.0339
SecretorStatus[Secretor]	0.6418	0.0406	0.6047	0.0380	0.6950	0.0342	0.5756	0.0339
BirthWt	0.1799	0.0114	0.1695	0.0106	0.6934	0.0341	0.5743	0.0338
TotalMorbidity	**2.3560**	-0.1492	**2.2197**	-0.1394	**2.1830**	-0.1073	**1.8082**	-0.1064
EBF Status @3mo [Y]	0.0102	-0.0006	-	-	0.1184	0.0058	-	-
EBF Status @3mo [N]	0.0102	0.0006	-	-	0.1184	-0.0058	-	-
EBF Status @6mo [Y]	-	-	**1.1365**	0.0714	-	-	**1.9025**	0.112
EBF Status @6mo[N]	-	-	**1.1365**	-0.0714	-	-	**1.9025**	-0.112
Fat	0.4437	-0.0281	0.418	-0.0263	0.3758	0.0185	0.3113	0.0183
Protein	**1.7450**	-0.1105	**1.6441**	-0.1033	**1.3017**	-0.064	**1.0782**	-0.0635

Lactose	0.4247	0.0269	0.4002	0.0251	**1.2565**	0.0618	**1.0408**	0.0613
True Protein	0.7910	-0.0501	0.7453	-0.0468	0.4031	-0.0198	0.3339	-0.0197
Fucosylated HMO	0.5187	0.0328	0.4887	0.0307	0.6052	0.0298	0.5013	0.0295
Sialylated HMO	0.3045	-0.0193	0.2869	-0.018	0.1606	0.0079	0.1330	0.0078
Undecorated HMO	0.5072	-0.0321	0.4779	-0.03	0.6095	-0.03	0.5049	-0.0297
Sia-fuc HMO	0.1014	0.0064	0.0955	0.006	0.7773	-0.0382	0.6438	-0.0379
Lactoferrin	0.0025	-0.0002	0.0024	-0.0002	0.6330	-0.0311	0.5243	-0.0309
IgA	0.4773	-0.0302	0.4497	-0.0282	**1.1962**	-0.0588	0.9909	-0.0583

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) and morbidity explanatory variables of interest (e.g., 9mo_3mo = 9mo growth outcome in relationship to 3mo dietary and morbidity variables); Maternal MUAC: 3rd trimester maternal MUAC measurement

Table 5.12. Summary of important (VIP > 1.0) PLS regression results ("real time" positive and negative predictors) of WHZ at 3, 6, 9, and 12 months of age

3mo		6mo		9mo		12mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Birth season (dry) EBF @ 3mo (N) Undec HMO	Parity EBF @ 3mo (Y) Protein TRP Fuc Sia-Fuc	Birth season (dry) EBF @ 6mo (Y) Birth Wt	Parity EBF @ 3mo (N) NonSecretor TotalMorbidity Sia HMO IgA	Birth season (dry)	Parity TotalMorbidity Protein	Birth season (dry) Lactose	Parity Maternal MUAC TotalMorbidity Protein IgA

Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Undec: Undecorated HMO class; Fuc: Fucosylated HMO class

Table 5.13. Summary of important (VIP > 1.0) PLS regression results ("extended" positive and negative predictors) of 3 and 6 month dietary conditions (maternal milk composition and EBF status) on 9 and 12 month WHZ

9mo_3mo		9mo_6mo		12mo_3mo		12mo_6mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Birth season (dry)	TotalMorbidity Protein	Birth season (dry) EBF @ 6mo (Y)	TotalMorbidity Protein	Birth season (dry) Lactose	Parity Maternal MUAC TotalMorbidity Protein IgA	Birth season (dry) EBF @ 6mo (Y) Lactose	Maternal MUAC Protein

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) and morbidity explanatory variables of interest (e.g., 9mo_3mo = 9mo growth outcome in relationship to 3mo dietary and morbidity variables); Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Maternal MUAC: 3rd trimester maternal MUAC measurement

HAZ

Infant HAZ across the first year of life was assessed in relationship to infant dietary, maternal, environmental, and sociodemographic factors using PLS regression models. Results are described below and detailed in **Table 5.14**. Summary tables of results can be found in **Table 5.15** and **Table 5.16**.

HAZ at 3 months of age

In the PLS regression assessing potential drivers of HAZ at the 3 month time point, the minimum root PRESS was 0.9982, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 37.6% and the percent variation explained for cumulative Y was 69.5%. The 1-component model showed 6 influential variables (VIP > 1.0) on infant HAZ at 3 months of age.

Milk composition and EBF status at 3 months had strong influence on HAZ at 3 months of age. Relative abundance of sialylated HMO had the strongest influence on 3 month HAZ, with greater relative abundance predicting lower HAZ by -0.1622 HAZs (VIP: 2.4024). Greater relative abundance of milk IgA also had a negative association with HAZ (VIP: 1.2261; B: -0.0828 HAZs). Higher HAZ was predicted by higher lactose content (VIP: 1.4037; B: 0.0948 HAZs). EBF to 3 months of age predicted higher HAZ by 0.0728 HAZs (VIP: 1.0776) relative to those no longer EBF at 3 months. Other infant and environmental variables also had significant influence on 3 month HAZ. Birth weight had a strong positive association with HAZ, with higher birth weight predicting higher HAZ by 0.1092 (VIP: 1.6165). Birth during the dry season predicted lower HAZ (VIP: 1.4356; B: -0.0969 HAZs) compared to birth during the wet season.

HAZ at 6 months of age

In the PLS regression assessing potential drivers of HAZ at the 3 month time point, the minimum root PRESS was 0.9861, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 36.8% and the percent variation explained for cumulative Y was 58.1%. The 1-component model showed 5 influential variables (VIP > 1.0) on infant HAZ at 6 months of age.

Milk composition and EBF status at 6 months both had strong influence on HAZ at 6 months of age. Relative abundance of sia-fuc HMO had the strongest influence on 6 month HAZ, with greater relative abundance predicting higher HAZ by 0.1520 HAZs (VIP: 1.7167). Greater milk protein content predicted lower 6 month HAZ by -0.1514

HAZs (VIP: 1.7100), and greater milk fat predicted higher HAZ by 0.0950 HAZs (VIP: 1.0734). EBF at 6 months of age predicted lower HAZ by -0.1475 HAZs (VIP: 1.6661). The environmental variable, birth season, also had strong influence on 6 month HAZ. Birth during the dry season predicted lower HAZ by -0.1307 HAZs (VIP: 1.4759).

HAZ at 9 months of age

"Real time" effects. In the PLS regression assessing potential drivers of HAZ at the 9 month time point, the minimum root PRESS was 0.9913, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 40.8% and the percent variation explained for cumulative Y was 68.2%. The 1-component model showed 5 influential variables (VIP > 1.0) on infant HAZ at 9 months of age.

Milk fat was the only dietary variable that was influential on HAZ at 9 months of age. Greater milk fat predicted greater HAZ by 0.1515 HAZs (VIP: 1.8036). Other influential variables included infant, environmental, and sociodemographic variables. Birth season had the strongest influence on 9 month HAZ, with birth during the dry season predicting lower HAZ by -0.1636 (VIP: 1.9478). Total morbidity occurrence was also a strong influential variable, with a greater number of morbidity occurrences predicting higher HAZ by 0.1180 HAZs (VIP: 1.4041). Birth weight had a positive influence on 9 month HAZ, with greater birth weight predicting higher HAZ by 0.1060 HAZs (VIP: 1.2616). Finally, household SEP influenced 9 month HAZ. Higher household SEP scores predicted lower HAZ by -0.0997 HAZs (VIP: 1.1873).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant HAZ at 9 months of age had a minimum root mean PRESS of 0.9955, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 45.7% and 69.9% for the percentage of variation explained for cumulative Y. The 1-component model showed 4 influential variables (VIP > 1.0) on infant HAZ at 9 months of age.

Milk fat at 3 months of lactation was the only dietary variable that had strong influence on HAZ at 9 months of age. Higher milk fat was associated with higher HAZ by 0.1517 HAZs (VIP: 1.8874). Infant and environmental variables also had strong influence on 9 month HAZ. Birth season had the strongest influence on HAZ, with birth during the dry season predicting lower HAZ by -0.1638 HAZs (VIP: 2.0383). Infant morbidity at 3 months was also

a strong predictor of HAZ at 9 months of age, with a greater number of morbidity occurrences predicting higher HAZ by 0.1181 HAZs (VIP: 1.4693). Finally, birth weight was positively associated with 9 month HAZ, with higher birth weight predicting higher HAZ by 0.1061 HAZs (VIP: 1.3202).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant HAZ at 9 months of age had a minimum root mean PRESS of 0.9548, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 40.8% and 68.9% for the percentage of variation explained for cumulative Y. The 1-component model showed 5 influential variables (VIP > 1.0) on infant HAZ at 9 months of age.

Milk composition and EBF status at 6 months had strong influence on HAZ at 9 months of age. Higher milk fat at 6 months of lactation predicted higher HAZ at 9 months of age by 0.1313 HAZs (VIP: 1.6964). EBF at 6 months of age predicted lower HAZ at 9 months by -0.1151 HAZs (VIP: 1.4868). Infant and environmental variables were also influential. Birth season had the strongest influence on 9 month HAZ, with birth during the dry season predicting lower HAZ by -0.1418 HAZs (VIP: 1.8321). A greater number of morbidity occurrences at 6 months of age predicted higher HAZ at 9 months by 0.1022 HAZs (VIP: 1.3207). Finally, birth weight had a positive association with 9 month HAZ, with higher birth weight predicting higher 9 month HAZ by 0.0919 HAZs (VIP: 1.1867).

HAZ at 12 months of age

In the PLS regression assessing potential drivers of HAZ at the 12 month time point, the minimum root PRESS was 0.9836, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 38.9% and the percent variation explained for cumulative Y was 58.1%. The 1-component model showed 4 influential variables (VIP > 1.0) on infant HAZ at 12 months of age.

None of the milk constituents at 12 months of lactation had significant influence on HAZ at 12 months of age. Instead, infant and maternal variables were influential. Total morbidity had the strongest influence on 12 month HAZ, with a greater number of morbidity occurrences predicting higher HAZ by 0.1138 HAZs (VIP: 1.7363). Infant sex also influenced 12 month HAZ, with female infants predicting lower HAZ by -0.0994 HAZs (VIP: 1.5172). Maternal Secretor status at 12 months also influenced HAZ. Non-Secretor status predicted higher HAZ by 0.1050 HAZs (VIP: 1.6022). Finally, higher birth weight predicted higher 12 month HAZ by 0.0931 HAZs (VIP: 1.4211).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant HAZ at 12 months of age had a minimum root mean PRESS of 0.9540, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 57.8% and 63.3% for the percentage of variation explained for cumulative Y. The 1-component model showed 4 influential variables (VIP > 1.0) on infant HAZ at 12 months of age

None of the dietary variables at 3 months predicted HAZ at 12 months of age. Instead, infant, maternal, and environmental variables were influential. Total morbidity at 3 months of age had the strongest influence on HAZ at 12 months of age, with a greater number of morbidity occurrences predicting higher HAZ by 0.1178 HAZs (VIP: 1.7895). Non-Secretor status predicted higher HAZ by 0.1087 HAZs (VIP: 1.6512). Female offspring predicted lower HAZ at 12 months by -0.1029 HAZs (VIP: 1.5637). Finally, birth weight also had a strong influence on 12 month HAZ, with a higher birth weight predicting higher HAZ by 0.0964 HAZs (VIP: 1.4646).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant HAZ at 12 months of age had a minimum root mean PRESS of 0.9842, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 48.8% and 70.1% for the percentage of variation explained for cumulative Y. The 1-component model showed 5 influential variables (VIP > 1.0) on infant HAZ at 12 months of age.

None of the milk constituents at 6 months of lactation had strong influence on HAZ at 12 months of age. EBF status at 6 months of age was a strong influence on HAZ, however, with continued EBF at 6 months predicting lower 12 month HAZ by -0.1018 HAZs (VIP: 1.5495). Other influential variables included maternal and infant variables. Total morbidity occurrence at 6 months of age had the strongest influence on 12 month HAZ, with a higher number of morbidity occurrences predicting higher HAZ by 0.1062 HAZs (VIP: 1.6164). Non-Secretor status predicted higher HAZ by 0.0980 HAZs (VIP: 1.4915). Female offspring were associated with lower HAZ by -0.0928 HAZs (VIP: 1.4124). Finally, birth weight had a strong positive association with 12 month HAZ, with higher birth weight predicting higher HAZ by 0.0869 HAZs (VIP: 1.3229).

Table 5.14. VIP statistics and model coefficients (B) from PLS regressions assessing “real time” and “extended” predictors of HAZ at 3, 6, 9, and 12 months of age for centered and scaled data

	"Real time" predictors of HAZ							
Time Point	**3mo**		**6mo**		**9mo**		**12mo**	
Term	**VIP**	B	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.047	0.0032	0.2968	-0.0263	0.8509	-0.0715	**1.5172**	-0.0994
Sex[M]	0.047	-0.0032	0.2968	0.0263	0.8509	0.0715	**1.5172**	0.0994
BirthSeason[Dry]	**1.4356**	-0.0969	**1.4759**	-0.1307	**1.9478**	-0.1636	0.303	0.0199
BirthSeason[Wet]	**1.4356**	0.0969	**1.4759**	0.1307	**1.9478**	0.1636	0.303	-0.0199
SEP Score	0.6536	-0.0441	0.3261	-0.0289	**1.1873**	-0.0997	0.5221	-0.0997
Parity	0.8588	0.058	0.2559	-0.0227	0.6714	0.0564	0.3048	0.02
Maternal MUAC	0.9785	0.0661	**1.0759**	-0.0952	0.5365	0.0451	0.9266	0.0607
SecretorStatus[Non-Secretor]	0.686	0.0463	0.1492	-0.0132	0.9319	0.0783	**1.6022**	0.105
SecretorStatus[Secretor]	0.686	-0.0463	0.1492	0.0132	0.9319	-0.0783	**1.6022**	-0.105
BirthWt	**1.6165**	0.1092	0.6585	0.0583	**1.2616**	0.106	**1.4211**	0.0931
EBF Status @3mo [Y]	**1.0776**	0.0728	-	-	-	-	-	-
EBF Status @3mo [N]	**1.0776**	-0.0728	-	-	-	-	-	-
EBF Status @6mo [Y]	-	-	**1.6661**	-0.1475	-	-	-	-
EBF Status @6mo[N]	-	-	**1.6661**	0.1475	-	-	-	-
TotalMorbidity	0.3256	-0.022	0.8186	0.0725	**1.4041**	0.118	**1.7363**	0.1138
Fat	0.1124	-0.0076	**1.0734**	0.095	**1.8036**	0.1515	0.0043	0.0003
Protein	0.2142	-0.0145	**1.71**	-0.1514	0.2103	0.0177	0.0108	-0.0007
Lactose	**1.4037**	0.0948	0.7606	0.0673	0.3923	-0.033	0.7531	-0.0494
True Protein	0.5953	-0.0402	0.8968	-0.0794	0.3288	0.0276	0.8123	-0.0532
Fucosylated HMO	0.3383	0.0228	0.8627	0.0764	0.1969	-0.0165	0.0921	0.006
Sialylated HMO	**2.4024**	-0.1622	0.5565	-0.0493	0.7546	-0.0634	0.6005	0.0394
Undecorated HMO	0.01	-0.0007	0.9498	-0.0841	0.2362	0.0198	0.1528	-0.01
Sia-fuc HMO	0.4789	0.0323	**1.7167**	0.152	0.4472	0.0376	0.5885	0.0386
Lactoferrin	0.8759	-0.0591	0.2503	-0.0222	0.1589	-0.0134	0.5654	0.0371
IgA	**1.2261**	-0.0828	0.114	0.0101	0.259	-0.0218	0.7	0.0459
	"Extended" predictors of HAZ							
Time Point	**9mo_3mo**		**9mo_6mo**		**12mo_3mo**		**12mo_6mo**	
Term	**VIP**	B	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.8905	-0.0716	0.8004	-0.062	**1.5637**	-0.1029	**1.4124**	-0.0928
Sex[M]	0.8905	0.0716	0.8004	0.062	**1.5637**	0.1029	**1.4124**	0.0928
BirthSeason[Dry]	**2.0383**	-0.1638	**1.8321**	-0.1418	0.3122	0.0206	0.282	0.0185
BirthSeason[Wet]	**2.0383**	0.1638	**1.8321**	0.1418	0.3122	-0.0206	0.282	-0.0185
SEP Score	0.2425	-0.0998	0.1168	-0.0865	0.5686	-0.1033	0.4169	-0.0931
Parity	0.7026	0.0565	0.6315	0.0489	0.3141	0.0207	0.2837	0.0186
Maternal MUAC	0.5614	0.0451	0.5046	0.0391	0.955	0.0629	0.8626	0.0567
SecretorStatus[Non-Secretor]	0.9752	0.0784	0.8765	0.0679	**1.6512**	0.1087	**1.4915**	0.098
SecretorStatus[Secretor]	0.9752	-0.0784	0.8765	-0.0679	**1.6512**	-0.1087	**1.4915**	-0.098
BirthWt	**1.3202**	0.1061	**1.1867**	0.0919	**1.4646**	0.0964	**1.3229**	0.0869
TotalMorbidity	**1.4693**	0.1181	**1.3207**	0.1022	**1.7895**	0.1178	**1.6164**	0.1062
EBF Status @3mo [Y]	0.0424	0.0034	-	-	0.5894	0.0388	-	-
EBF Status @3mo [N]	0.0424	-0.0034	-	-	0.5894	-0.0388	-	-
EBF Status @6mo [Y]	-	-	**1.4868**	-0.1151	-	-	**1.5495**	-0.1018
EBF Status @6mo[N]	-	-	**1.4868**	0.1151	-	-	**1.5495**	0.1018
Fat	**1.8874**	0.1517	**1.6964**	0.1313	0.0044	0.0003	0.004	0.0003
Protein	0.2200	0.0177	0.1978	0.0153	0.0112	-0.0007	0.0101	-0.0007

Lactose	0.4106	-0.033	0.369	-0.0286	0.7762	-0.0511	0.7011	-0.0461
True Protein	0.3441	0.0276	0.3093	0.0239	0.8372	-0.0551	0.7562	-0.0497
Fucosylated HMO	0.2061	-0.0166	0.1852	-0.0143	0.0949	0.0062	0.0857	0.0056
Sialylated HMO	0.7897	-0.0635	0.7098	-0.055	0.6188	0.0407	0.5590	0.0367
Undecorated HMO	0.2472	0.0199	0.2222	0.0172	0.1575	-0.0104	0.1422	-0.0093
Sia-fuc HMO	0.4680	0.0376	0.4207	0.0326	0.6066	0.0399	0.5479	0.0360
Lactoferrin	0.1663	-0.0134	0.1495	-0.0116	0.5827	0.0384	0.5263	0.0346
IgA	0.2710	-0.0218	0.2436	-0.0189	0.7214	0.0475	0.6516	0.0428

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) and morbidity explanatory variables of interest (e.g., 9mo_3mo = 9mo growth outcome in relationship to 3mo dietary and morbidity variables); Maternal MUAC: 3rd trimester maternal MUAC measurement

Table 5.15. Summary of important (VIP > 1.0) PLS regression results ("real time" positive and negative predictors) of HAZ at 3, 6, 9, and 12 months of age

3mo		6mo		9mo		12mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Birth season (wet) Birth Wt EBF @ 3mo (Y) Lactose	Birth season (dry) EBF @ 3mo (N) Sia HMO IgA	Birth season (wet) EBF @ 6mo (N) Fat Sia-Fuc HMO	Birth season (dry) Maternal MUAC EBF @ 6mo (Y) Protein	Birth season (wet) Birth Wt TotalMorbidity Fat	Birth season (dry)	Sex (male) NonSecretor Birth Wt TotalMorbidity	Sex (female) Secretor

Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Sia: Sialylated HMO class

Table 5.16. Summary of important (VIP > 1.0) PLS regression results ("extended" positive and negative predictors) of 3 and 6mo dietary variables (maternal milk composition and EBF status) on 9 and 12mo HAZ

9mo_3mo		9mo_6mo		12mo_3mo		12mo_6mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Birth season (wet) Birth Wt TotalMorbidity Fat	Birth season (dry)	Birth season (wet) Birth Wt TotalMorbidity EBF @6mo (N) Fat	Birth season (dry) EBF @6mo (Y)	Sex (male) NonSecretor	Sex (female) Secretor	Sex (male) NonSecretor Birth wt TotalMorbidity EBF @6mo (N)	Sex (female) Secretor EBF @6mo (Y)

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) and morbidity explanatory variables of interest (e.g., 9mo_3mo = 9mo growth outcome in relationship to 3mo dietary and morbidity variables); Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable

WAZ

Infant WAZ across the first year of life was assessed in relationship to infant dietary, maternal, environmental, and sociodemographic factors using PLS regression models. Results are described below and detailed in **Table 5.17**. Summary tables of results can be found in **Table 5.18** and **Table 5.19.**

WAZ at 3 months of age

In the PLS regression assessing potential drivers of WAZ at the 3 month time point, the minimum root PRESS was 0.9809, which is considered significant, and the minimizing number of factors was 1. The percent variation

explained for cumulative X was 41.4% and the percent variation explained for cumulative Y was 88.4%. The 1-component model showed 7 influential variables (VIP > 1.0) on infant WAZ at 3 months of age.

Milk composition, but not EBF status, at 3 months had a strong influence on WAZ at 3 months of age. Sia HMO had the strongest influence on WAZ, with greater relative abundance of Sia predicting lower WAZ by -0.1596 WAZs (VIP: 2.1648). Lactose also had a strong association with WAZ, with greater lactose content predicting higher WAZ by 0.1491 WAZs (VIP: 2.0228). Lower WAZ at 3 months of age was predicted by greater protein and TRP content by -0.0937 WAZs (VIP: 1.2708) and -0.1078 WAZs (VIP: 1.4623), respectively. Greater relative abundances of LF and IgA also predicted lower WAZ, by -0.0951 WAZs (VIP: 1.2907) and -0.0846 WAZs (VIP: 1.1482), respectively. The only non-dietary variable that had strong influence on WAZ at 3 months of age was birth weight. A higher birth weight predicted a higher WAZ ay 3 months by 0.1211 WAZs (VIP: 1.6430).

WAZ at 6 months of age

In the PLS regression assessing potential drivers of WAZ at the 6 month time point, the minimum root PRESS was 0.9879, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 48.1% and the percent variation explained for cumulative Y was 66.2%. The 1-component model showed 7 influential variables (VIP > 1.0) on infant WAZ at 6 months of age.

Milk composition, but not EBF status, at 6 months was influential on WAZ at 6 months of age. Milk protein at 6 months of lactation had a negative association with 6 month WAZ, with greater milk protein content predicting lower WAZ by -0.1077 WAZs (VIP: 1.3016). Greater relative abundances of sialylated HMO and IgA predicted lower WAZ by -0.0835 WAZs (VIP: 1.0086) and -0.0920 WAZs (VIP: 1.1116), respectively. The other influential variables were maternal, infant, and environmental. Birth weight had the strongest association with 6 month WAZ, with a higher birth weight predicting higher WAZ by 0.2164 WAZs (VIP: 2.6145). Birth during the dry season also predicted higher WAZ at 6 months (VIP: 1.2369; B: 0.1024 WAZs). Maternal parity and maternal nutritional status during the 3rd trimester both had negative associations with 6 month WAZ. Specifically, higher maternal parity predicted lower WAZ by -0.1131 WAZs (VIP: 1.3667), and higher maternal MUAC measurements predicted lower WAZ by -0.0870 WAZs (VIP: 1.0507).

WAZ at 9 months of age

"Real time" effects. In the PLS regression assessing potential drivers of WAZ at the 9 month time point, the minimum root PRESS was 0.9778, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 40.0% and the percent variation explained for cumulative Y was 52.6%. The 1-component model showed 6 influential variables (VIP > 1.0) on infant WAZ at 9 months of age.

Milk composition at 9 months of lactation influenced WAZ at 9 months of age. Milk protein content had the strongest influence on 9 month WAZ, with greater protein content predicting lower WAZ by -0.1358 WAZs (VIP: 1.9859). Greater relative abundance of sialylated HMO also predicted lower WAZ, by -0.0868 WAZs (VIP: 1.2694). Greater fat content in maternal milk predicted higher WAZ at 9 months by 0.0942 WAZs (VIP: 1.3791). Other maternal and infant variables were also influential. Higher birth weight predicted higher WAZ at 9 months by 0.1187 WAZs (VIP: 1.7365). Infant morbidity occurrence by 9 months of age had a negative influence on 9 month WAZ, with a greater number of morbidity occurrences predicting lower WAZ by -0.1042 (VIP: 1.5246). Finally, higher parity also predicted lower WAZ by -0.1155 WAZs (VIP: 1.6893).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant WAZ at 9 months of age had a minimum root mean PRESS of 0.9963, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 36.9% and 65.1% for the percentage of variation explained for cumulative Y. The 1-component model showed 7 influential variables (VIP > 1.0) on infant WAZ at 9 months of age.

Milk composition at 3 months of lactation, but not EBF status at 3 months, was influential on WAZ at 9 months of age. Milk protein content at 3 months had the strongest influence on 9 month WAZ, with greater protein content predicting lower WAZ by -0.1348 WAZs (VIP: 2.0772). Greater relative abundance of sialylated HMO at 3 months of lactation predicted lower WAZ at 9 months by -0.0862 WAZs (VIP: 1.3277). Higher fat content in maternal milk at 3 months of lactation predicted higher WAZ at 9 months by 0.0936 WAZs (VIP: 1.4425). The other influential variables were infant, maternal, and sociodemographic. Infant birth weight had a strong positive association with WAZ at 9 months, with higher birth weight predicting higher WAZ by 0.1179 WAZs (VIP: 1.8163). Infant morbidity at 3 months had a negative association with 9 month WAZ, with a greater number of morbidity occurrence predicting

lower WAZ by -0.1035 WAZs (VIP: 1.5947). Higher maternal parity predicted lower WAZ at 9 months (VIP: 1.7670; B: -0.1147 WAZs) and higher household SEP predicted lower WAZ as well (VIP: 1.2407; B: -0.0805 WAZs).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant WAZ at 9 months of age had a minimum root mean PRESS of 0.9943, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 40.0% and 54.1% for the percentage of variation explained for cumulative Y. The 1-component model showed 7 influential variables (VIP > 1.0) on infant WAZ at 9 months of age.

Milk composition, but not EBF status, at 6 months was influential on WAZ at 9 months of age. Milk protein content at 6 months of lactation had the strongest association with WAZ at 9 months of age, with greater protein content predicting lower WAZ by -0.1340 WAZs (VIP: 2.0737). Greater relative abundance of sia HMO also predicted lower WAZ (VIP: 1.3255; B:-0.0856 WAZs). Higher fat content in maternal milk at 6 months of lactation predicted higher WAZ at 9 months of age by 0.0930 WAZs (VIP: 1.4401). Other influential variables include infant, maternal, and sociodemographic. Infant birth weight had a strong influence on 9 month WAZ, with a higher birth weight predicting higher WAZ at 9 months by 0.1171 WAZs (VIP: 1.8135). Infant morbidity at 6 months had a negative association with WAZ at 9 months, with greater number of morbidity occurrences at 6 months predicting lower 9 month WAZ by -0.1028 WAZs (VIP: 1.5920). Higher maternal parity predicted lower WAZ by -0.1139 WAZs (VIP: 1.7641) as did higher household SEP (VIP: 1.2386; B: -0.0800).

WAZ 12 months of age

"Real time" effects. In the PLS regression assessing potential drivers of WAZ at the 12 month time point, the minimum root PRESS was 0.9611, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 36.6% and the percent variation explained for cumulative Y was 52.7%. The 1-component model showed 4 influential variables (VIP > 1.0) on infant WAZ at 12 months of age.

Milk composition at 12 months of lactation had strong influence on WAZ at 12 months of age. Greater protein and TRP content both predicted lower WAZ, by -0.1102 WAZs (VIP: 1.2094) and -0.0956 WAZs (VIP: 1.0488), respectively. In addition to these dietary variables, infant and environmental variables were also influential. The strongest influence on 12 month WAZ was birth weight. Higher birth weight predicted higher 12 month WAZ by

0.1797 WAZs (VIP: 1.9725). Seasonality also influenced WAZ at 12 months. Birth during the dry season predicted higher WAZ at 12 months compared to birth during the wet season by 0.1734 WAZs (VIP: 1.9031).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant WAZ at 12 months of age had a minimum root mean PRESS of 0.9702, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 42.1% and 56.2% for the percentage of variation explained for cumulative Y. The 1-component model showed 5 influential variables (VIP > 1.0) on infant WAZ at 12 months of age.

Milk composition, but not EBF status, at 3 months influenced WAZ at 12 months of age. Specifically, greater protein and TRP content in maternal milk predicted lower WAZ by -0.1021 WAZs (VIP: 1.2448) and -0.0885 WAZs (VIP: 1.0796), respectively. Other influential variables were infant, environmental, and sociodemographic. Infant birth weight had the strongest influence on WAZ at 12 months of age. Higher birth weight predicted higher 12 month WAZ by 0.1665 WAZs (VIP: 2.0304). Household SEP had a negative influence on 12 month WAZ, with higher SEP predicting lower WAZ by -0.0834 WAZs (VIP: 1.0168). Birth during the dry season predicted higher WAZ at 12 months of age by 0.1607 WAZs (VIP: 1.9590).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant WAZ at 12 months of age had a minimum root mean PRESS of 0.9956, which was considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 23.9% and 49.8% for the percentage of variation explained for cumulative Y. The 1-component model showed 5 influential variables (VIP > 1.0) on infant WAZ at 12 months of age.

Milk composition, but not EBF status, at 6 months influenced WAZ at 12 months of age. Specifically, higher protein and TRP content in maternal milk at 6 months of lactation predicted lower WAZ at 12 months by -0.1102 WAZs (VIP: 1.2417) and -0.0955 WAZs (VIP: 1.0768), respectively. The other influential variables were infant, environmental, and sociodemographic. Infant birth weight had the strongest influence on 12 month WAZ, with higher birth weight predicting higher WAZ by 0.1797 WAZs (VIP: 2.0252). Birth during the dry season predicted higher WAZ by 0.1734 WAZs (VIP: 1.9540) compared to birth during the wet season. Finally, higher household SEP predicted lower WAZ at 12 months of age by -0.0900 WAZs (VIP: 1.0142).

Table 5.17. VIP statistics and model coefficients (B) from PLS regressions assessing “real time” and “extended” predictors of WAZ at 3, 6, 9, and 12 months of age for centered and scaled data

	"Real time" predictors of WAZ							
Time Point	**3mo**		**6mo**		**9mo**		**12mo**	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.5893	-0.0434	0.634	-0.0525	0.7396	-0.0506	0.9173	-0.0836
Sex[M]	0.5893	0.0434	0.634	0.0525	0.7396	0.0506	0.9173	0.0836
BirthSeason[Dry]	0.5114	-0.0377	**1.2369**	0.1024	0.2782	0.0190	**1.9031**	0.1734
BirthSeason[Wet]	0.5114	0.0377	**1.2369**	-0.1024	0.2782	-0.0190	**1.9031**	-0.1734
SEP Score	0.2904	-0.0214	0.1475	0.0122	0.1861	-0.0811	0.9878	-0.0900
Parity	0.5482	-0.0404	**1.3667**	-0.1131	**1.6893**	-0.1155	0.6836	-0.0623
Maternal MUAC	0.5681	0.0419	**1.0507**	-0.0870	0.5842	-0.0399	0.5115	-0.0466
SecretorStatus[Non-Secretor]	0.9083	0.0670	0.9882	-0.0818	0.2820	0.0193	0.83	0.0756
SecretorStatus[Secretor]	0.9083	-0.0670	0.9882	0.0818	0.2820	-0.0193	0.83	-0.0756
BirthWt	**1.6430**	0.1211	**2.6145**	0.2164	**1.7365**	0.1187	**1.9725**	0.1797
TotalMorbidity	0.0285	-0.0021	0.8109	-0.0671	**1.5246**	-0.1042	0.4952	-0.0451
EBF Status @3mo [Y]	0.3761	0.0277	-	-	-	-	-	-
EBF Status @3mo [N]	0.3761	-0.0277	-	-	-	-	-	-
EBF Status @6mo [Y]	-	-	0.3041	0.0252	-	-	-	-
EBF Status @6mo[N]	-	-	0.3041	-0.0252	-	-	-	-
Fat	0.4498	-0.0332	0.9929	0.0822	**1.3791**	0.0943	0.42	0.0383
Protein	**1.2708**	-0.0937	**1.3016**	-0.1077	**1.9859**	-0.1358	**1.2094**	-0.1102
Lactose	**2.0228**	0.1491	0.7405	0.0613	0.1239	0.0085	0.407	0.0371
True Protein	**1.4623**	-0.1078	0.6155	-0.0509	0.7539	-0.0515	**1.0488**	-0.0956
Fucosylated HMO	0.4132	-0.0305	0.4998	0.0414	0.4118	0.0282	0.7018	0.0639
Sialylated HMO	**2.1648**	-0.1596	**1.0086**	-0.0835	**1.2694**	-0.0868	0.8237	0.0751
Undecorated HMO	0.7396	0.0545	0.3878	-0.0321	0.3508	-0.024	0.7751	-0.0706
Sia-fuc HMO	0.0767	-0.0057	0.0024	0.0002	0.6481	0.0443	0.061	-0.0056
Lactoferrin	**1.2907**	-0.0951	0.5011	-0.0415	0.0776	-0.0053	0.0391	-0.0036
IgA	**1.1482**	-0.0846	**1.1116**	-0.0920	0.9440	-0.0645	0.4163	-0.0379
	"Extended" predictors of WAZ							
Time Point	**9mo_3mo**		**9mo_6mo**		**12mo_3mo**		**12mo_6mo**	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.7736	-0.0502	0.7723	-0.0499	0.9442	-0.0774	0.9418	-0.0836
Sex[M]	0.7736	0.0502	0.7723	0.0499	0.9442	0.0774	0.9418	0.0836
BirthSeason[Dry]	0.2910	0.0189	0.2905	0.0188	**1.9590**	0.1607	**1.9540**	0.1734
BirthSeason[Wet]	0.2910	-0.0189	0.2905	-0.0188	**1.9590**	-0.1607	**1.9540**	-0.1734
SEP Score	**1.2407**	-0.0805	**1.2386**	-0.08	**1.0168**	-0.0834	**1.0142**	-0.0900
Parity	**1.767**	-0.1147	**1.7641**	-0.1139	0.7037	-0.0577	0.7019	-0.0623
Maternal MUAC	0.6111	-0.0397	0.6101	-0.0394	0.5265	-0.0432	0.5251	-0.0466
SecretorStatus[Non-Secretor]	0.2950	0.0191	0.2945	0.0190	0.8544	0.0701	0.8522	0.0756
SecretorStatus[Secretor]	0.2950	-0.0191	0.2945	-0.0190	0.8544	-0.0701	0.8522	-0.0756
BirthWt	**1.8163**	0.1179	**1.8133**	0.1171	**2.0304**	0.1665	**2.0252**	0.1797
TotalMorbidity	**1.5947**	-0.1035	**1.592**	-0.1028	0.5098	-0.0418	0.5085	-0.0451
EBF Status @3mo [Y]	0.1089	0.0071	-	-	0.6120	0.0502	-	-
EBF Status @3mo [N]	0.1089	-0.0071	-	-	0.6120	-0.0502	-	-
EBF Status @6mo [Y]	-	-	0.2250	-0.0145	-	-	0.6564	0.0582
EBF Status @6mo[N]	-	-	0.2250	0.0145	-	-	0.6564	-0.0582
Fat	**1.4425**	0.0936	**1.4401**	0.0930	0.4323	0.0355	0.4312	0.0383
Protein	**2.0772**	-0.1348	**2.0737**	-0.134	**1.2448**	-0.1021	**1.2417**	-0.1102

Lactose	0.1296	0.0084	0.1293	0.0084	0.4190	0.0344	0.4179	0.0371
True Protein	0.7886	-0.0512	0.7872	-0.0509	**1.0796**	-0.0885	**1.0768**	-0.0955
Fucosylated HMO	0.4307	0.0280	0.4300	0.0278	0.7224	0.0592	0.7205	0.0639
Sialylated HMO	**1.3277**	-0.0862	**1.3255**	-0.0856	0.8479	0.0695	0.8458	0.0750
Undecorated HMO	0.3670	-0.0238	0.3663	-0.0237	0.7979	-0.0654	0.7958	-0.0706
Sia-fuc HMO	0.6779	0.0440	0.6767	0.0437	0.0628	-0.0052	0.0627	-0.0056
Lactoferrin	0.0811	-0.0053	0.0810	-0.0052	0.0402	-0.0033	0.0401	-0.0036
IgA	0.9874	-0.0641	0.9857	-0.0637	0.4286	-0.0351	0.4275	-0.0336

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) and morbidity explanatory variables of interest (e.g., 9mo_3mo = 9mo growth outcome in relationship to 3mo dietary and morbidity variables); Maternal MUAC: 3rd trimester maternal MUAC measurement

Table 5.18. Summary of important (VIP > 1.0) PLS regression results ("real time" positive and negative predictors) of WAZ at 3, 6, 9, and 12 months of age

3mo		6mo		9mo		12mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Birth Wt Lactose	Protein TRP Sia HMO LF IgA	Birth season (wet) Birth Wt	Birth season (dry) Parity Maternal MUAC Protein Sia HMO IgA	Birth Wt Fat	Parity TotalMorbidity Protein Sia HMO	Birth season (dry) Birth Wt	Birth season (wet) Protein TRP

Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Sia: Sialylated HMO class; Maternal MUAC: 3rd trimester maternal MUAC measurement; LF: Lactoferrin

Table 5.19. Summary of important (VIP > 1.0) PLS regression results ("extended" positive and negative predictors) of 3 and 6mo dietary conditions (maternal milk composition and EBF status) on 9 and 12mo WAZ

9mo_3mo		9mo_6mo		12mo_3mo		12mo_6mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Birth Wt Fat	Parity TotalMorbidity Protein Sia HMO SEP	Birth Wt Fat	Parity TotalMorbidity Protein Sia HMO SEP	Birth season (dry) Birth Wt	Birth season (wet) Protein TRP SEP	Birth season (dry) Birth Wt	Birth season (wet) SEP Protein TRP

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) and morbidity explanatory variables of interest (e.g., 9mo_3mo = 9mo growth outcome in relationship to 3mo dietary and morbidity variables); Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Sia: Sialylated HMO class

Mixed effects models of growth outcomes

WHZ

Mixed effects model analyses showed that WHZ across the first year of life was significantly predicted by infant morbidity. Specifically, a greater number of cumulative infant morbidity occurrences significantly predicted lower WHZ by -0.07 WHZs (95% CIs: -0.12, -0.01; P=0.0186). The random variable, Subject ID, was also a significant predictor of WHZ (B: 0.63 WHZs; 95% CIs: 0.24, 1.01; P=0.0013). None of the dietary variables, including maternal milk composition or EBF duration, were significant predictors in the statistical model.

HAZ

Mixed effects model analyses showed that Subject ID significantly predicted HAZ (B: 0.88 HAZs; 95% CIs: 0.35, 1.4 HAZ; P=0.0011). None of the dietary variables, including maternal milk composition or EBF duration, were significant predictors in the statistical model.

WAZ

Birth weight, protein, Subject ID Mixed effects model analyses showed that infant birth weight was a significant predictor of WAZ across the first year of life. Specifically, a higher birth weight significantly predicted higher WAZ by 1.12 WAZs (95% CIs: 0.19, 2.05 WAZ; P=0.0196). Milk protein content, but no other milk constituent, significantly predicted WAZ, with lower WAZ predicted by higher protein content (B: -0.94 WAZs, 95% CIs: -1.77, -0.11 WAZ; P=0.0274). Subject ID was also a significant predictor of WAZ (B: 0.46 WAZs; 95% CIs: 0.18, 0.74 WAZ; P=0.0014). EBF duration was not a significant predictor of WAZ.

Table 5.20. Mixed effects model results of potential predictors of growth outcomes across the first 12 months of life

	WHZ			HAZ			WAZ		
Term	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**	**B**	**LB, UB**	**P**
Intercept	-2	-7.53, 3.53	0.4709	-1.86	-7.98, 4.25	0.5424	-1.92	-6.48, 2.63	0.3994
Time point[3]	0.32	-0.1, 0.75	0.1367	0.07	-0.36, 0.5	0.7443	0.21	-0.12, 0.55	0.2075
Time point[6]	0.12	-0.08, 0.32	0.2234	0.13	-0.07, 0.32	0.2009	0.11	-0.05, 0.26	0.1721
Time point[9]	-0.07	-0.28, 0.13	0.4803	-0.04	-0.24, 0.16	0.6938	-0.07	-0.23, 0.08	0.3526
Time point[12] *Referent*	-	-	-	-	-	-	-	-	-
Sex[F]	-0.04	-0.37, 0.29	0.8009	-0.07	-0.45, 0.31	0.6984	-0.05	-0.33, 0.23	0.7019
BirthSeason[Dry]	0.22	-0.18, 0.62	0.2645	-0.12	-0.58, 0.33	0.5867	0.06	-0.27, 0.4	0.6959
SEP Score	0.02	-0.11, 0.15	0.7566	-0.05	-0.2, 0.1	0.4747	-0.01	-0.12, 0.1	0.8231
Parity	-0.03	-0.19, 0.12	0.6763	-0.06	-0.24, 0.12	0.5006	-0.06	-0.19, 0.08	0.3981
Maternal MUAC	-0.04	-0.18, 0.1	0.5833	0.03	-0.14, 0.19	0.7258	-0.01	-0.13, 0.11	0.8179
SecretorStatus[Non-Secretor]	-0.01	-0.26, 0.24	0.9310	0.03	-0.23, 0.29	0.8408	0.01	-0.19, 0.21	0.9201
BirthWt	0.95	-0.15, 2.06	0.0892	0.75	-0.52, 2.02	0.2371	1.12	0.19, 2.05	**0.0196***
TotalMorbidity	-0.07	-0.12, -0.01	**0.0186***	0.01	-0.05, 0.07	0.6948	-0.04	-0.09, 0.0	0.0594
EBF Duration (mo)	-0.01	-0.24, 0.21	0.8927	0.01	-0.24, 0.27	0.9253	0	-0.19, 0.19	0.9896
Fat	0.07	-0.02, 0.17	0.1405	-0.04	-0.14, 0.05	0.3739	0.03	-0.05, 0.1	0.4704
Protein	-1.03	-2.1, 0.04	0.0586	-0.36	-1.41, 0.69	0.4963	-0.94	-1.77, -0.11	**0.0274***
Lactose	-0.04	-0.28, 0.2	0.7262	0.03	-0.2, 0.26	0.8025	-0.01	-0.19, 0.17	0.9133
True Protein	0.2	-0.87, 1.27	0.7104	-0.13	-1.17, 0.92	0.8115	0.01	-0.82, 0.84	0.9746
Fucosylated HMO	0.01	-0.01, 0.03	0.2592	-0.01	-0.03, 0.01	0.4482	0	-0.01, 0.02	0.9576
Sialylated HMO	0.11	-0.05, 0.27	0.1907	-0.01	-0.17, 0.15	0.9119	0.01	-0.11, 0.13	0.8769
Sia-fuc HMO	-0.14	-0.35, 0.07	0.2011	0.15	-0.06, 0.35	0.1634	0.06	-0.11, 0.22	0.4994
Lactoferrin	0.11	-0.4, 0.61	0.6740	-0.11	-0.6, 0.38	0.6672	-0.06	-0.45, 0.33	0.7593
IgA	-0.01	-1, 0.97	0.9800	-0.52	-1.49, 0.46	0.2937	-0.36	-1.13, 0.41	0.3542
Subject ID	0.63	0.24, 1.01	**0.0013***	0.88	0.35, 1.4	**0.0011***	0.46	0.18, 0.74	**0.0014***

B: Coefficient estimate; 95% CIs: Confidence Intervals (Lower Bound, Upper Bound); P: P-value; *P<.0001

Fecal pH across the first 12 months of life

The mean (SD) fecal pH at 3, 6, 9, and 12 months of age in this population was 4.96 (±0.6), 5.24 (±0.9), 5.46 (±0.9), and 5.75 (±0.9), respectively (**Table 5.21**). Though not significantly different between each other, the average fecal pH at 3 and 6 months of age were significantly lower compared to the 9 and 12 time points ($P<0.05$). Fecal pH was not significantly different based on EBF duration (<6mo or ≥6mo).

Table 5.21. Infant fecal pH by collection time point and according to exclusive breastfeeding duration

Age (mo)	Value	HERO-G Subsample (N=194)	EBF <6mo (N=135)	EBF ≥6mo (N=59)
3	Mean	4.96	5.01	4.87
	SD	0.65	0.68	0.55
	N	107	74	33
6	Mean	5.24	5.27	5.30
	SD	0.93	0.86	1.08
	N	88	59	29
9	Mean	5.46	5.47	5.45
	SD	0.94	0.92	1.10
	N	104	74	30
12	Mean	5.75	5.78	5.69
	SD	0.93	0.96	0.87
	N	92	64	28

SD: Standard deviation

Predictors of fecal pH

Infant fecal pH across the first year of life was assessed in relationship to infant dietary, maternal, environmental, and sociodemographic factors using PLS regression models. Results are described below and detailed in **Table 5.22.** Summary tables of results can be found in **Table 5.23.** and **Table 5.24.**

Fecal pH at 3 months of age

In the PLS regression assessing potential drivers of fecal pH at the 3 month time point, the minimum root PRESS was 0.9767, which is considered significant, and the minimizing number of factors was 1. The percent

variation explained for cumulative X was 49.6% and the percent variation explained for cumulative Y was 84.9%. The 1-component model showed 5 influential variables (VIP > 1.0) on infant fecal pH at 3 months of age.

Maternal milk composition, but not EBF status, at 3 months was influential on infant fecal pH at 3 months of age. Greater protein and TRP content in maternal milk at 3 months of lactation predicted higher fecal pH at 3 months of age by 0.1734 pH (VIP: 1.5307) and 0.1527 pH (VIP: 1.3481), respectively. Lactose had a negative association with fecal pH, where higher lactose content predicted lower fecal pH by -0.1561 pH (VIP: 1.3776). The other influential variables were maternal and sociodemographic. Household SEP had the strongest influence on fecal pH at 3 months of age, with higher SEP predicting lower fecal pH by -0.3052 pH (VIP: 2.6933). Higher maternal MUAC during the 3rd trimester predicted lower fecal pH by -0.1962 pH (VIP: 1.7317).

Fecal pH at 6 months of age

In the PLS regression assessing potential drivers of infant fecal pH at the 6 month time point, the minimum root PRESS was 0.9751, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 72.1% and the percent variation explained for cumulative Y was 66.2%. The 1-component model showed 5 influential variables (VIP > 1.0) on infant fecal pH at 6 months of age.

Milk HMOs and HMGP were influential on fecal pH at 6 months of age. Specifically, relative abundance of fucosylated HMO and LF had negative associations with fecal pH. Greater relative abundance of fucosylated HMO predicted lower fecal pH by -0.0987 pH (VIP: 1.4804). Similarly, greater relative abundance of LF predicted lower fecal pH by -0.0798 pH (VIP: 1.1976). Greater relative abundance of undecorated HMO predicted higher fecal pH by 0.1076 pH (VIP: 1.6139). In addition to these dietary factors, infant sex influenced infant fecal pH at 6mo, with female infants predicting lower fecal pH by -0.0870 pH (VIP: 1.3056). Finally, maternal Secretor status had the strongest influence on fecal pH at 6 months of age, with non-Secretor status predicting higher fecal pH by 0.1338 pH (VIP: 2.0070).

Fecal pH at 9 months of age

"Real time" effects. In the PLS regression assessing potential drivers of infant fecal pH at the 9 month time point, the minimum root PRESS was 0.9665, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 51.6% and the percent variation explained for cumulative

Y was 72.6%. The 1-component model showed 6 influential variables (VIP > 1.0) on infant fecal pH at 9 months of age.

Milk composition had an important influenced infant fecal pH at 9 months of age. Specifically, higher fat content in maternal milk predicted higher fecal pH by 0.1857 pH (VIP: 1.3421). Milk protein content influenced fecal pH, with greater protein content predicting higher fecal pH 0.2341 (VIP: 1.6918). Milk HMOs were also influential. Greater relative abundance of fucosylated HMO and sia-fuc HMO predicted higher fecal pH by 0.1444 pH (VIP: 1.0440) and 0.2448 (VIP: 1.7694), respectively. Undecorated HMO had an inverse relationship with fecal pH, with greater relative undecorated HMO predicting lower fecal pH by -0.1612 pH (VIP: 1.1649). Maternal parity had the strongest influence on infant fecal pH at 9 months, with greater parity predicting higher fecal pH by 0.3242 pH (VIP: 2.3434). Higher maternal MUAC during the 3rd trimester predicted higher fecal pH by 0.1690 pH (VIP: 1.2218).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant fecal pH at 9 months of age had a minimum root mean PRESS was 0.9981, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 25.6% and 84.0% for the percentage of variation explained for cumulative Y. The 1-component model showed 8 influential variables (VIP > 1.0) on infant fecal pH at 9 months of age.

Maternal parity was the strongest predictor of infant fecal pH at 9 months of age, with higher parity predicting higher fecal pH by 0.3465 pH (VIP: 2.3083). Greater relative abundances of fucosylated HMO and sia-fuc HMO in milk collected at 3 months of age predicted higher fecal pH at 9 months of age by 0.1544 pH (VIP: 1.0284) and 0.2616 pH (VIP: 1.7429), respectively. Relative abundance of undecorated HMO at 3 months of lactation had a negative association with infant fecal pH at 9 months of age (VIP: 1.1474; B: -0.1722). Greater milk fat and protein content at 3 months of lactation predicted higher infant fecal pH at 9 months of age by 0.1984 (VIP: 1.3220) and 0.2501 pH (VIP: 1.6665), respectively. Maternal nutritional status during the 3rd trimester was also positively associated with infant fecal pH at 9 months of age (VIP: 1.2035; B: 0.1806). Finally, infants still EBF at 3 months of age predicted lower fecal pH at 9 months of age by -0.1700 pH (VIP: 1.1324).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant fecal pH at 9 months of age had a minimum root mean PRESS of 0.9984, which is considered significant, and the

minimizing number of factors was 1. The percent variation explained for cumulative X was 28.3% and 70.8% for the percentage of variation explained for cumulative Y. The 1-component model showed 6 influential variables (VIP > 1.0) on infant fecal pH at 9 months of age.

Maternal parity had the strongest influence on infant fecal pH at 9 months of age, with higher parity predicting higher fecal pH by 0.3206 pH (VIP: 2.4608). Relative abundances of fucosylated HMO and sia-fuc HMO in maternal milk collected at 6 months of lactation had positive associations with infant fecal pH at 9 months of age (fucosylated – VIP: 1.0963; B: 0.1428; sia-fuc – VIP: 1.8580; B: 0.2421). Greater relative abundances of undecorated HMO at 6 months of lactation predicted lower infant fecal pH by -0.1594 pH (VIP: 1.2232) at 9 months of age. Greater milk fat and protein content at 6 months of lactation predicted higher fecal pH at 9 months of age by 0.1836 pH (VIP: 1.4093) and 0.2314 pH (VIP: 1.7765), respectively. Finally, maternal nutrition during the 3rd trimester also had a positive association with infant fecal pH at 9 months of life (VIP: 1.2830; B: 0.1672).

Fecal pH at 12 months of age

"Real time" effects. In the PLS regression assessing potential drivers of fecal pH at the 12 month time point, the minimum root PRESS was 0.9844, which is considered significant, and the minimizing number of factors was 1. The percent variation explained for cumulative X was 38.4% and the percent variation explained for cumulative Y was 54.6%. The 1-component model showed 5 influential variables (VIP > 1.0) on infant fecal pH at 12 months of age.

Greater relative abundance of milk IgA at 12 months of lactation predicted higher fecal pH by 0.1448 pH (VIP: 1.9291). Lactose content had a negative association with fecal pH, with higher lactose predicting lower fecal pH by -0.0787 pH (VIP: 1.0481). Lower fecal pH was predicted by greater relative abundance of fucosylated HMO by -0.1042 (VIP: 1.3888), and higher fecal pH was predicted by greater relative abundance undecorated HMO by 0.1047 pH (VIP: 1.3953). Household SEP had the strongest influence on infant fecal pH at 12 months of age, with higher SEP predicting higher fecal pH by 0.1908 pH (VIP: 2.5425).

"Extended" effects of dietary variables and morbidity occurrence at 3 months of age. PLS regression incorporating milk composition and infant EBF status at 3 months of age to assess their relationships to infant fecal pH at 12 months of age had a minimum root mean PRESS of 0.9988 and the minimizing number of factors was 1. The percent variation explained for cumulative X was 21.6% and 36.9% for the percentage of variation explained for

cumulative Y. The 1-component model showed 6 influential variables (VIP > 1.0) on infant fecal pH at 12 months of age.

Household SEP had the strongest influence on infant fecal pH at 12 months of age (VIP: 2.5263; B: 0.1836). Relative abundances of undecorated HMO and milk IgA collected in milk at 3 months of lactation had positive associations with infant fecal pH at 12 months of age (undecorated – VIP: 1.3864; B: 0.1007; IgA – VIP: 1.9168; B: 0.1393). Relative abundances of fucosylated HMO in milk collected at 3 months of age had a negative association with infant fecal pH at 12 months of age (VIP: 1.3799; B: -0.1003). Lactose content at 3 months of age also had a negative relationship with infant fecal pH, with greater lactose content predicting lower fecal pH by -0.0757 pH (VIP: 1.0414).

"Extended" effects of dietary variables and morbidity occurrence at 6 months of age. PLS regression incorporating milk composition and infant EBF status at 6 months of age to assess their relationships to infant fecal pH at 12 months of age had a minimum root mean PRESS was 0.9912 and the minimizing number of factors was 1. The percent variation explained for cumulative X was 27.8% and 34.4% for the percentage of variation explained for cumulative Y. The 1-component model showed that household SEP, milk lactose content at 6 months of lactation, and relative abundances of fucosylated HMO, sialylated HMO, and milk IgA at 6 months of lactation were influential (VIP > 1.0) on infant fecal pH at 12 months of age.

Household SEP had the strongest influence on infant fecal pH at 12 months of age in this PLS regression (VIP: 2.6718; B: 0.1897). Relative abundance of milk IgA at 6 months of lactation also had a strong association with infant fecal pH at 12 months of age, with greater relative abundance of IgA predicting higher fecal pH by 0.1439 pH (VIP: 2.0272). Relative abundances of sialylated HMO and undecorated HMO in milk collected at 6 months of lactation both had positive associations with infant fecal pH at 12 months of age (sialylated – VIP: 1.025; B: 0.0728; undecorated – VIP: 1.4663; B: 0.1041). Relative abundance of fucosylated HMO at 6 months of lactation predicted lower infant fecal pH by -0.1036 pH (VIP: 1.4594). Lactose content in milk collected at 6 months of lactation predicted lower infant fecal pH at 12 months of age by -0.0782 pH (VIP: 1.1014).

Table 5.22. Results of PLS regression assessing potential "real time" and "extended" predictors of infant fecal pH across the first 12 months of life

	"Real time" predictors of fecal pH							
Time Point	3mo		6mo		9mo		12mo	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.2293	0.0260	**1.3056**	-0.087	0.4606	0.0637	0.2331	0.0175
BirthSeason[Dry]	0.8846	0.1002	0.2340	0.0156	0.0361	-0.005	0.7539	-0.0566
Maternal MUAC	**1.7317**	-0.1962	0.8936	-0.0596	**1.2218**	0.1690	0.2937	-0.0220
SecretorStatus[Non-Secretor]	0.6469	-0.0733	**2.0070**	0.1338	0.1177	0.0163	0.3605	-0.0271
SEP Score	**2.6933**	-0.3052	0.6141	-0.0409	0.4624	0.0640	**2.5425**	0.1908
Parity	0.1109	0.0126	0.3925	-0.0262	**2.3434**	0.3242	0.0004	0
EBF Status @3mo [Y]	0.2516	0.0285	-	-	-	-	-	-
EBF Status @3mo [N]	0.2516	-0.0285	-	-	-	-	-	-
EBF Status @6mo [Y]	-	-	0.4537	-0.0302	-	-	-	-
EBF Status @6mo[N]	-	-	0.4537	0.0302	-	-	-	-
Fat	0.5851	0.0663	0.6575	-0.0438	**1.3421**	0.1857	0.7639	0.0573
Protein	**1.5307**	0.1734	0.3326	0.0222	**1.6918**	0.2341	0.7174	-0.0538
Lactose	**1.3776**	-0.1561	0.5077	0.0338	0.2259	-0.0313	**1.0481**	-0.0787
True Protein	**1.3481**	0.1527	0.3138	0.0209	0.9184	0.1271	0.1129	0.0085
Fucosylated HMO	0.2399	-0.0272	**1.4804**	-0.0987	**1.0440**	0.1444	**1.3888**	-0.1042
Sia-fuc HMO	0.6806	-0.0771	0.3633	-0.0242	**1.7694**	0.2448	0.3758	-0.0282
Sialylated HMO	0.7548	0.0855	0.5257	-0.035	0.1033	-0.0143	0.9754	0.0732
Undecorated HMO	0.1789	0.0203	**1.6139**	0.1076	**1.1649**	-0.1612	**1.3953**	0.1047
Lactoferrin	0.1070	-0.0121	**1.1976**	-0.0798	0.4452	-0.0616	0.2117	0.0159
IgA	0.7528	-0.0853	0.3821	0.0255	0.0522	0.0072	**1.9291**	0.1448
	"Extended" predictors of fecal pH							
Time Point	9mo_3mo		9mo_6mo		12mo_3mo		12mo_6mo	
Term	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**	**VIP**	**B**
Sex[F]	0.4537	0.0681	0.4836	0.063	0.2317	0.0168	0.2450	0.0174
BirthSeason[Dry]	0.0356	-0.0053	0.0379	-0.0049	0.7491	-0.0544	0.7923	-0.0563
Maternal MUAC	**1.2035**	0.1806	**1.2830**	0.1672	0.2918	-0.0212	0.3086	-0.0219
SecretorStatus[Non-Secretor]	0.1160	0.0174	0.1236	0.0161	0.3582	-0.0260	0.3788	-0.0269
SEP Score	0.4555	0.0684	0.4855	0.0633	**2.5263**	0.1836	**2.6718**	0.1897
Parity	**2.3083**	0.3465	**2.4608**	0.3206	0.0004	0	0.0004	0
EBF Status @3mo [Y]	**1.1324**	-0.1700	-	-	**1.0587**	0.0769	-	-
EBF Status @3mo [N]	**1.1324**	0.1700	-	-	**1.0587**	-0.0769	-	-
EBF Status @6mo [Y]	-	-	0.1567	0.0204	-	-	0.0973	-0.0069
EBF Status @6mo[N]	-	-	0.1567	-0.0204	-	-	0.0973	0.0069
Fat	**1.3220**	0.1984	**1.4093**	0.1836	0.7590	0.0551	0.8027	0.0570
Protein	**1.6665**	0.2501	**1.7765**	0.2314	0.7128	-0.0518	0.7539	-0.0535
Lactose	0.2226	-0.0334	0.2373	-0.0309	**1.0414**	-0.0757	**1.1014**	-0.0782
True Protein	0.9047	0.1358	0.9644	0.1256	0.1122	0.0081	0.1186	0.0084
Fucosylated HMO	**1.0284**	0.1544	**1.0963**	0.1428	**1.3799**	-0.1003	**1.4594**	-0.1036
Sia-fuc HMO	**1.7429**	0.2616	**1.858**	0.2421	0.3734	-0.0271	0.3949	-0.0280
Sialylated HMO	0.1017	-0.0153	0.1084	-0.0141	0.9692	0.0704	**1.025**	0.0728
Undecorated HMO	**1.1474**	-0.1722	**1.2232**	-0.1594	**1.3864**	0.1007	**1.4663**	0.1041
Lactoferrin	0.4386	-0.0658	0.4675	-0.0609	0.2103	0.0153	0.2224	0.0158
IgA	0.0514	0.0077	0.0548	0.0071	**1.9168**	0.1393	**2.0272**	0.1439

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) variables of interest (e.g.,

9mo_3mo = 9mo fecal pH in relationship to 3mo dietary variables); Maternal MUAC: 3rd trimester maternal MUAC measurement

Table 5.23. Summary of important (VIP > 1.0) PLS regression results ("real time" positive and negative predictors) of infant fecal pH at 3, 6, 9, and 12 months of age

3mo		6mo		9mo		12mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Protein TRP	Maternal MUAC SEP Lactose	Sex (male) NonSecretor Undec HMO	Sex (female) Secretor Fuc HMO LF	Maternal MUAC Parity Fat Protein Fuc HMO Sia Fuc HMO	Undec HMO	SEP Undec HMO IgA	Lactose Fuc HMO

Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Undec: Undecorated HMO class; Fuc: Fucosylated HMO class; Sia: Sialylated HMO class; LF: Lactoferrin

Table 5.24. Summary of important (VIP > 1.0) PLS regression results ("extended" positive and negative effects) of 3 and 6 month dietary variables (maternal milk composition and EBF status) on 9 and 12 month fecal pH

9mo_3mo		9mo_6mo		12mo_3mo		12mo_6mo	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Maternal MUAC Parity EBF @ 3mo (N) Fat Protein Fuc HMO Sia-Fuc HMO	EBF @ 3mo (Y) Undec HMO	Maternal MUAC Parity Fat Protein Fuc HMO Sia-Fuc HMO	Undec HMO	SEP EBF @ 3mo (Y) Undec HMO IgA	EBF @ 3mo (N) Lactose Fuc HMO	SEP Sia HMO Undec HMO IgA	Fuc HMO Lactose

"Extended" effects time points denoted as: Xmo_Ymo, where X represents the collection time point of the output of interest and where Y represents the collection time point of the dietary (EBF status and milk composition) and morbidity explanatory variables of interest (e.g., 9mo_3mo = 9mo growth outcome in relationship to 3mo dietary and morbidity variables); Positive: Explanatory variable with important (VIP > 1.0) positive association with output variable; Negative: Explanatory variable with important (VIP > 1.0) negative association with output variable; Undec: Undecorated HMO class; Fuc: Fucosylated HMO class; Sia: Sialylated HMO class; Maternal MUAC: 3rd trimester maternal MUAC measurement

Mixed effects model results (fecal pH)

Results from the mixed effects model showed that, after adjusting for sample collection time point, infant sex, season of birth, household SEP, maternal parity, maternal nutritional status during pregnancy, and maternal Secretor status, significant predictors of infant fecal pH across the first year of life include relative abundances of sialylated HMO and milk IgA. Greater relative concentrations of sialylated HMO significantly predicted lower fecal pH by -0.23 pH (95% CIs: -0.48, 0.01; P=0.0476). Higher fecal pH was predicted by greater relative abundance of milk IgA by 0.93 pH (95% CIs: 0.3, 3.55; P=0.0211). EBF duration was not a significant predictor of fecal pH. Full model results are detailed in **Table 5.25**.

Table 5.25. Mixed effects model results of potential predictors of fecal pH across the first 12 months of life

Term	B	LB, UB	P
Intercept	8.63	1.62, 15.65	**0.0168***
Time point[3]	0.05	-0.54, 0.64	0.8707
Time point[6]	0.03	-0.4, 0.45	0.8978
Time point[9]	0.03	-0.41, 0.47	0.8878
Time point[12] *Referent*	-	-	-
Sex[F]	0.27	-0.02, 0.57	0.0640
BirthSeason[Dry]	0.07	-0.3, 0.43	0.7136
SEP Score	-0.03	-0.14, 0.07	0.5321
Parity	0.02	-0.11, 0.14	0.7536
Maternal MUAC	-0.07	-0.19, 0.04	0.2139
SecretorStatus[Non-Secretor]	-0.08	-0.41, 0.26	0.6523
EBF Duration (mo)	0.04	-0.13, 0.21	0.6427
Fat	-0.04	-0.23, 0.14	0.6348
Protein	-0.3	-2.27, 1.66	0.7589
Lactose	-0.01	-0.63, 0.6	0.9630
True Protein	0.77	-1.34, 2.87	0.4680
Fucosylated HMO	-0.02	-0.05, 0	0.1045
Sialylated HMO	-0.23	-0.48, 0.01	**0.0476***
Sia-fuc HMO	0.07	-0.29, 0.44	0.6903
Lactoferrin	-0.55	-1.57, 0.47	0.2882
IgA	0.93	0.3, 3.55	**0.0211***
Subject ID	-0.1	-0.32, 0.12	0.3870

B: Coefficient estimate; 95% CIs: Confidence Intervals (Lower Bound, Upper Bound); P: P-value; *P<.0001

Population comparison of infant fecal pH in the last century

Data were compiled from available publications on breastfed infant fecal pH published within the last century in order to compare to the results of our measures of intestinal acidity in the HERO-G cohort. A total of 19 publications were included in the present analysis. The average rural Gambian infant fecal pH is significantly lower (more acidic) compared to infants of similar age in other populations (P<0.0001) (**Table 5.26**). The average infant fecal pH within the last 50 years is 5.9, whereas rural Gambian infants of similar age have an average pH of 5.1. Additionally, of the available publications, only three were conducted in low-income populations. As such, further research in LMIC populations is required in order to evaluate whether infants from The Gambia are significantly different from populations under similar pressures.

Table 5.26. Population comparison of infant fecal pH in the last century

Country	Year	N	Age (mo)	Fecal pH	SD	Author
The Gambia	Present	121	3	4.98	0.64	Present Analysis
The Gambia	Present	97	6	5.24	0.91	Present Analysis
The Gambia	Present	115	9	5.49	0.95	Present Analysis
The Gambia	Present	104	12	5.75	0.93	Present Analysis
The Gambia	Present	84	18	5.92	0.88	Present Analysis
The Gambia	Present	63	24	5.87	0.86	Present Analysis
Argentina	1992	7	1-5	5.8	0.6	Ogawa et al.
Bangladesh	2019	100	12-18	5.84	1.11	Hossain et al.
Canada	1924	NR	0.5	4.7-5.1	NR	Tisdall
Denmark	1942	17	0.5-5.5	5.5	0.56	Uldall
Germany	1917	NR	NR	4.6-5.6	NR	Eitel
Germany	1921	NR	NR	4.8-5.6	NR	Freudenberg & Heller
Germany	2005	21	2-3	5.8	NR	Knol et al.
Germany	2008	32	1	6.38	0.1	Mohan et al.
Japan	1960	9	NR	5.3	0.25	Nagai
Japan	2016	15	1	5.9	0.6	Matsuki
UK	1971	10	NR	5.2	0.43	Bullen & Willis
UK	1977	13	NR	5.1	NR	Bullen et al.
UK	1982	17	1.5	5.9	NR	Simhon et al.

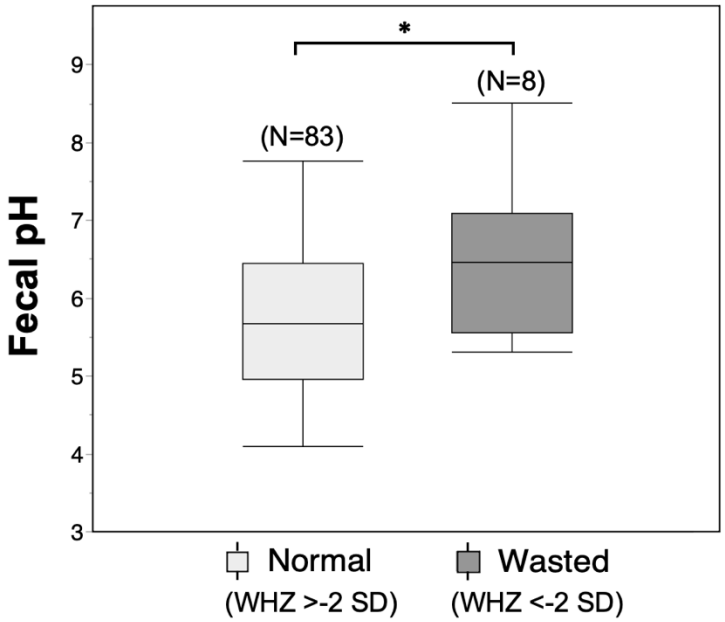
Country	Year	N	Age (mo)	Fecal pH	SD	Author
UK	1989	38	1	6.18	0.67	Balmer & Wharton
US	1926	19	NR	4.88	0.22	Norton
US	1952	7	0.5-3	5.5	NR	Barbero et al.
US	1955	71	NR	5.4	NR	Pratt & Read
US	2011	33	1-3	6.41	0.11	Holscher et al.
US	2017	18	1	5.97	0.57	Frese et al.

NR: Not reported

Fecal pH and growth

Wilcoxon rank sums tests showed that infant fecal pH was significantly higher (P=0.0380) in wasted infants (N=8) compared to those with normal WHZ (N=83) at 12 months of age. On average, wasted infants had a fecal pH of 6.5 (±1.0) and those of normal WHZ had an average pH of 5.7 (±0.9) (**Figure 5.2**). There were no significant differences in infant fecal pH according to WHZ growth outcomes at 3, 6, or 9 months of age.

Figure 5.2. Fecal pH in wasted (WHZ < -SD) compared to normal (WHZ > -2SD) infants at 12 months of age



*P<0.05

There were no significant differences in infant fecal pH according to WAZ growth outcomes at 3, 6, 9, or 12 months of age. Similarly, there was no significant difference in fecal pH at any time point for stunted versus normal

height-for-age infants. Mean (SD) fecal pH values across the first 12 months of life and according to WHZ, HAZ, and WAZ group are detailed in **Table 5.27**.

Table 5.27. Mean (SD) fecal pH across the first 12 months of life and according to growth outcome

	WHZ				**HAZ**				**WAZ**			
	Normal		*Wasted*		*Normal*		*Stunted*		*Normal*		*Underweight*	
Age (mo)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)
3	102	4.96 (0.6)	3	5.27 (0.6)	94	4.96 (0.6)	11	5.02 (0.7)	95	4.95 (0.6)	10	5.16 (0.7)
6	83	5.25 (0.9)	4	5.70 (0.6)	81	5.30 (1.0)	6	4.90 (0.5)	78	5.28 (1.0)	9	5.16 (0.7)
9	93	5.46 (1.0)	10	5.57 (0.9)	93	5.48 (1.0)	10	5.34 (0.8)	87	5.43 (1.0)	15	5.61 (0.9)
12	83	5.68 (0.9)	8	**6.52 (1.0)***	75	5.75 (0.9)	17	5.75 (1.1)	75	5.70 (0.9)	17	5.97 (1.2)

Significantly higher fecal pH indicated by boldface font and gray cell color; *P<0.05

DISCUSSION

The interrelationships between diet, health, and growth in early life are complex. In this Chapter, I examined associations between infant diet (maternal milk composition and exclusive breastfeeding duration), health status (morbidity occurrence), and linear growth outcomes (WHZ, HAZ, WAZ) across the first 12 months of life in the HERO-G subsample. Through these analyses, I identified "real time" and "extended" effects of breastfeeding practices, maternal milk composition, and infant morbidity on growth at 3, 6, 9, and 12 months of age. This work demonstrates the persistent contributions of first foods on offspring outcomes.

EBF duration had a significant positive association with growth outcomes in the PLS regression models at 3 and 6 months of life in this population. However, it is important to note that the coefficients for the influence of EBF on growth outcomes were sometimes small. Several other studies investigating the effect of exclusive breastfeeding to 6 months of age on infant growth in LMICs also report limited benefit to growth outcomes during early life, aligning with these results[264,562–565]. The majority of existing studies examining the impact of exclusive breastfeeding to 6 months of age have been conducted in affluent populations or in urbanized regions of LMICs[264,561,581–584]. Malnutrition in the form of overweight and obesity are generally more prevalent in these populations compared to growth stunting or faltering, as is use of commercial formulas during the transition from exclusive breastfeeding[539,585–587].

Maternal milk composition also impacted growth outcomes during the first year of life in this subsample. First, protein was a significant driver of WAZ across the first year of life in the PLS regression models and mixed effects model, where greater protein content in maternal milk predicted lower WAZ. This challenges findings from other studies, which show that dietary protein has a positive correlation with body weight[531,588]. In fact, high concentrations of protein are commonly used in infant formulas to supplement the diet of infants born prematurely and those with low birth weights[589]. Feeding frequency has been shown to impact the timing and amount of milk protein produced in maternal milk, which has been suggested to influence infant appetite[590]. One possible explanation relates to the general trend in growth outcomes in rural Gambia over the last four decades, where infants are born small and continue to fall away from the WHO growth centiles over the first two years of life[261]. The growth trends for the HERO-G subsample followed these trends. While infants are still exclusively breastfed and thus receiving protection from infectious via maternal milk, rural Gambian infant weight shows early growth catchup[261]. The trend is clear in WHZ as there is a decline in length during this time relative to body weight. Milk protein was not negatively associated with infant WHZ at 3 or 6 months of age in the HERO-G subsample, which encapsulates the period in which infants are exclusively or primarily receiving maternal milk in this population. Along these same lines, infant HAZ was not strongly influenced by milk protein at any time point in this analysis. Milk fat content had a positive relationship with HAZ but not WHZ or WAZ in the present analysis. Higher total lipid content in human milk has been linked to lower infant weight, adiposity and BMI gain between months 3 and 12 months of age[452].

Here, a relationship was also found between infant WHZ and fucosylated HMO. This has been documented in two other studies in human populations. Saben et al. (2021) found that sialylated HMOs (specifically infant intakes of 3'FL, 3'SL, 6'SL, disialyllacto-N-tetraose, disialyllacto-N-hexaose, and total acidic HMOs) were positively associated with infant growth during the first 6 months of life in a US population[591]. Charbonneau et al. (2016) found that Malawian mothers whose infants were stunted produced milk with significantly lower concentrations of fucosylated, sialylated, and total HMOs compared to those whose infants were growing well[592]. One report has demonstrated a causal microbiota-dependent association between sialylated HMOs and infant growth outcomes through an improved ability to utilize nutrients for anabolism[592]. Mouse and piglet models showed that, in offspring colonized with feces from 6 month old growth stunted infants from Malawi, diets supplemented with sialylated HMOs resulted in improved growth; they noted an increase in body weight gain, lean mass, changes in bone morphology, and metabolic shifts indicative of appropriate substrate utilization patterns[592]. The animals that were not colonized

with the feces from the stunted infant did not have the same results. This model was representative of the transitional period from exclusive breastfeeding to introduction of complementary foods, thus providing particularly useful context for the purposes of this dissertation[592]. It has also been suggested that HMO composition may impact infant feeding behaviors, thereby directly influencing growth outcomes through changes in caloric intake[593]. Menzel et al (2021) found that associations between HMOs (including sialylated HMOs) and infant growth outcomes may extend beyond periods of exclusive breastfeeding, highlighting the importance of evaluating both maternal and infant factors in assessments of underlying associations[594]. Previous studies have shown that shifts in maternal milk HMO composition is associated with infant health in The Gambia[437]. Future studies should further examine individual HMO structures in relationship to infant health and growth outcomes. A relationship between the relative abundance of LNFP I was higher in infants experiencing morbidity compared to those who were not[437]. Relative abundance of LNFP I was also predictive of HAZ at 20 weeks of age. This may suggest involvement of this particular HMO structure in protecting the infant from infection and thus supporting growth outcomes[437]. Available evidence to determine potential mechanisms for infant growth promotion through HMO consumption is scarce. The data that do exist show strong evidence that the infant gut microbiome plays a mediatory role in the process, as HMOs are indigestible to human infants and are instead critical to the establishment of a healthy gut microbiota composition[570,591].

Here, lower WHZ was significantly predicted by greater cumulative infant morbidity in the mixed effects model analyses, and at 6, 9, and 12 months in the PLS regressions. Cumulative morbidity was also predictive of HAZ and WAZ at certain time points. Previous studies in the West Kiang region of The Gambia have found evidence of a significant negative impact of morbidity on infant growth, though there is some evidence that EBF infants can be buffered from these effects[69,264,482]. One study in this region reports that children weaned before 3 months of age showed a trend towards slowed growth and lower weight in the month following weaning compared to EBF infants, but the difference was insignificant[156]. Moore et al. (2001) report that diarrhea burdens were significantly linked with growth faltering, even after adjusting for numerous variables, including nutritional status during infancy[595].

Statistical models showed an important influence of birth season on growth outcomes for at least two time points for each growth index (and at all 4 time points for WHZ). Previous work in this region has assessed the role of seasonality on early life immune programming and subsequent effects on health/growth outcomes[126]. Chapter 4 detailed the associations between birth season and infant morbidity occurrence across the first year of life, and reports findings which suggest that extended EBF duration protects offspring from morbidity in this environment.

Between 3 and 12 months of life in this subsample, morbidity had a negative impact on infant growth outcomes, with those experiencing a greater number of morbidity events having lower WHZ. These findings likely indicate that chronic or more frequent symptomatic illnesses divert resources away from growth and towards physiological systems for self-maintenance. This may be viewed as an evolutionary strategy to increase survivorship and maximize fitness under challenging environmental conditions. Many other studies have also found significant evidence linking morbidity to compromised growth during early life[264,596,597]. In the established undernutrition-infection cycle (detailed in Chapter 1), infections increase nutrient needs and can reduce appetite, and poor nutritional intake can compromise immune function, increasing disease susceptibility[547,598]. Particularly in resource-limited populations, effects from an increase in energy expenditure towards immune function may result in poor linear growth outcomes. In the Greater Banjul and Upper River Regions, one study found strong evidence of an association between undernutrition and both severe and non-severe pneumonia, and a significant link between severe pneumonia and severe stunting[504]. Gastrointestinal illness, which was common in this subsample (see Chapter 4), has also been shown to influence growth outcomes in this region, and has been tied to gut microbiota composition during early life. Because cumulative morbidity had a significant negative impact on WHZ in particular, an interrelationship between these variables and seasonal pressures should be assessed in greater detail. Further investigation of the impact of the specific morbidities experienced (e.g., GI, respiratory, dermatological) and/or delta morbidity between time points rather than cumulative morbidity may further clarify the role of morbidity on growth outcomes. Ultimately, there is great complexity in the interplay between environmental pressures, immune function, intestinal ecology, and early life growth outcomes.

Microbial landscapes in the intestines operate best under certain ecological conditions. Of interest in this dissertation was the response of intestinal pH to diet and the relationships between pH and growth outcomes. First, I did not find a significant difference in real time infant fecal pH based on exclusive breastfeeding duration. Because components of human milk are the sole form of food for bifidobacteria, and because nearly all infants were breastfed across the first year of life (Chapter 2), I interpret this lack of association as attributable to the continued and extended duration of breastfeeding in this population. In other studies examining infant fecal pH, there is trend towards non-exclusively breastfed and non-breastfed infants to have more alkaline gut environments compared to those exclusively or partially breastfed. As such, it was unlikely that I would see a significant difference between fecal pH of the two groups. Additionally, previous research examining the bifidobacteria abundance in the rural Gambian infant found

that around 70% of the gut microbiota was composed of bifidobacteria, demonstrating a high prevalence of this fermentative species across the board. Future analyses of infant fecal pH in relationship to infant gut microbiota composition would greatly benefit a more nuanced evaluation of the relationships between infant diet and intestinal health in this population. With lower gut pH linked to reduced morbidity and improved growth outcomes, fecal pH may have promise as a non-invasive marker of intestinal health, and could perhaps be used to flag poor growth outcomes. There was evidence of an extended effect of EBF duration on fecal pH, where infants EBF at 3 months of age predicted lower fecal pH at 9 and 12 months of age. Longer-term impacts of early life intestinal pH as it relates to specific durations of EBF have not yet been investigated in the literature. This exploratory work sets the stage for future investigations.

Greater abundance of IgA in maternal milk predicted higher infant fecal pH. IgA has anti-inflammatory effects and is implicated in the efficiency of mucosal barriers throughout the body, including the GI tract[362–369]. It is possible that IgA functions in a responsive model, though much of the literature suggests it plays more of a protective role in infant immune defense. Also, milk IgA has been observed to be elevated during periods of both maternal and infant infection. High relative abundance of IgA may reflect presence of a current morbidity, which could result in higher fecal pH. As discussed in earlier sections, a lower gut pH creates a more hospitable physical landscape for bifidobacteria, a key gut microbe involved in infant immune system development. A more acidic gut also prevents colonization by certain pathogens, providing numerous health benefits to offspring such as reduced inflammation and improved immune function[207]. Loss or absence of bifidobacteria in the infant gut significantly decreases this organic acid production, which results in elevated fecal pH, which is linked to poor health and growth outcomes[208,209]. In the present analysis, I observed that infant fecal pH was significantly predicted by the total number of morbidity events experienced by an infant across the first 24 months of life. In particular, a greater number of GI morbidity events predicts a significantly higher mean fecal pH in this population. Examining these results in a temporal manner with clinic data on infant and maternal morbidities may be useful in understanding the timing of milk IgA concentrations elevating and symptoms of intestinal inflammation. Additional real time markers of inflammation, as opposed to cumulative number of symptomatic morbidities, would pair well with future research on gut microbiota composition in this cohort.

There was a significant difference in fecal pH at between wasted and those of normal WHZ at 12 months of age in the HERO-G subsample. No significant relationships were found between HAZ or WAZ at any time point.

Hossain et al. (2019) recently found evidence of relationships between fecal pH and growth outcomes, though the study population included only those who were stunted and at risk of stunting, whereas this analysis included a range of growth outcomes. Additional research is warranted on the matter to evaluate fecal pH as a flag for infants at risk of growth faltering. Measuring fecal pH could also present an effective and sustainable option in the field, where transport and storage limit opportunities for microbiome research in anthropological fieldwork in many rural population.

Through the comparison of 19 publications, the average healthy breastfed infant fecal pH has increased from 5.0 to 6.0 between 1926 and 2017 in high- and middle-income populations[208,209]. Paralleling the shift in pH, research has shown a concurrent reduction, or in some cases, loss of *Bifidobacterium* in the infant gut, along with a significant increase in autoimmune disorders over the last century[208,209,599]. These marked shifts suggest that low pH may be a critical condition necessary in preventing invasion of harmful bacteria and fostering growth of beneficial bacteria (particularly *Bifidobacterium)* in the infant gut[208,209]. Therefore, lower gut pH may be an important promoter of offspring survival due to its involvement in pathogen prevention and protection through modulation of gut microbiota[600]. Environmental enteropathy in rural Gambian infants and children, as evidenced by the structural and immunological of the gastrointestinal tract, has been suggested to be a possible advantage to local environmental conditions[479]. Constant exposure to fecal-oral contamination and/or pathogenic infiltration into the gut mucosa may result in an individual remaining in an inflammatory, hyperimmune state may be the body's adaptive response to such challenges. The fluctuations in disease related to the stark seasonality in The Gambia are not commonly seen in other regions of the world, so it is possible that such a distinction in gut pH is further evidence of a maintained advantageous state in response to local challenges. Ultimately, further investigations into the intestinal ecology of rural Gambian infants – including research on gut morphology, inflammatory responses, and acidity conditions within the gut – are required to make stronger inferences about the relationships between the intestinal landscape during early life and its possible roots in developmental plasticity as an evolutionary adaptation in response to the challenging environment.

First foods – including maternal milk and introduction of non-breast milk foods – communicate important information about the local environment and present immunological challenges that can help 'teach' the immune system how to optimally respond to such pressures. It is possible that EBF duration relates to health and growth outcomes depending on presence or absence of symptoms. Infant health and growth attainment may influence a mother's decision to introduce complementary foods to her infant earlier; however, existing research provides mixed results on the directionality of these relationships. For example, mothers may cease EBF or alter breastfeeding or

complementary feeding practices because their infants are ill (perhaps hospitalized), type of illness, or perception that maternal milk is not meeting the infant's needs[158]. Others may wean infants perceived as healthy (e.g., fewer occurrences of morbidity) at earlier ages, which may result in increased incidence of morbidity due to reduced immunological protection from maternal milk. Infants perceived as growing well may receive NBMFs earlier if they seem demand more feeding; mothers may also EBF for longer durations if they perceive breastfeeding as deterministic to healthy growth[264]. The variation in perceptions may bias the order of causality and lead to inaccurate estimation of the immediate protective effects of EBF.

CONCLUSION

The goal of Chapter 5 was to incorporate findings and data from Chapters 2-4 to assess the influence of early life diet (maternal milk composition, exclusive breastfeeding duration), infant morbidity occurrence, environmental factors, sex differences, maternal nutritional status during pregnancy, and demographic characteristics on infant growth outcomes (WHZ, HAZ, and WAZ) across the first 12 months of life in the HERO-G subsample. Growth outcomes, which followed patterns documented in rural Gambia over the last four decades, were impacted by infant morbidity occurrence and maternal milk composition in the present analyses.

Humans, like other animals, have the ability to alter development, physiology, growth, and behavior in response to environmental conditions. Tradeoffs (e.g., growth versus self-maintenance or investment in current offspring versus future offspring) are physiologically computed by taking into account energy balance and environmental stress, and subsequent biological and/or behavioral alterations are initiated in response. Using findings from Chapters 2-4, Chapter 5 found evidence of tradeoffs between immune function and growth outcomes across the first year of life in the rural Gambian environment. Weight, as opposed to height (representative of skeletal growth), is prone to fluctuation, which can reflect "real time" shifts in nutritional environment or food security (amongst other possible explanatory factors). Evidence of the tradeoff between self-maintenance and growth was more apparent in WHZ and WAZ outcomes in the HERO-G subsample, suggesting shorter-term buffering or modifications to physiological and developmental systems. Statistical models also found that breastfeeding practices (often influenced by factors such as annual shifts in maternal agricultural workloads in this region – see Chapter 2) impacted WHZ and HAZ outcomes during the first 6 months of life.

Maternal energy investment in milk composition throughout the course of lactation contributes to offspring growth, survival, and future reproductive success of the mother. The mechanisms in place to allow for such effects are influenced, in part, by infant gut microbiota and their interactions with dietary variables. In the exploratory analysis of infant fecal pH across the first year of life, the intestinal landscape appears to be influenced by maternal milk components and breastfeeding practices, along with its own links to infant growth outcomes. Several milk constituents were also influential on growth outcomes, both in real time and as extended effects. The strong impact of individual variation in maternal milk profiles and infant health and growth outcomes in this rural Gambian environment speaks to an underlying complexity and persistent contribution of first foods on offspring outcomes.

Using symptomatic infant morbidity occurrence as a proxy for immune activation, the present results likely indicate that chronic or more frequent symptomatic illnesses divert resources away from growth and towards physiological systems for self-maintenance. This could represent a strategy to increase survivorship and maximize fitness in response to challenging environmental pressures. Future investigations may consider assessing milk micronutrients, gut microbiota composition, biological markers of inflammation, exposure or risk of exposure to pathogens, and more detailed assessments of household SEP, all of which have been shown to impact growth or physiological systems related to growth in existing studies. Finally, infant fecal pH was linked to certain growth outcomes in this population, and a larger sample size will be helpful in identifying any population-level patterns. This work sets a strong foundation for such future investigations.

CHAPTER 6. MAJOR CONCLUSIONS AND FUTURE DIRECTIONS

PROJECT GOALS

The major goal of this project was to investigate the impact of first foods – including maternal milk and non-breast milk foods – on early life health and growth in a population under considerable environmental and physiological stress. I utilized data from a subsample of 194 mother-infant pairs from the larger HERO-G study in the West Kiang region of The Gambia to investigate my research questions.

SUMMARY OF MAIN RESULTS

In Chapter 2, I characterized infant breastfeeding and complementary feeding practices and assessed determinants of exclusive breastfeeding duration over the first year of life in the HERO-G subsample. This analysis provided a broad understanding of infant feeding practices across 194 mother-infant pairs. The average exclusive breastfeeding duration in this subsample was 5.0 (±1.5) months, slightly lower than the WHO recommended duration of 6 months. Most infants (69.6%) were exclusively breastfed for less than 6 months. On average, infants born in May – shortly before the start of the wet season – began receiving non-breast milk before the end of the wet season and had the shortest exclusive breastfeeding duration (3.5 ±1.9 months) compared to those born in any other month (except October). A more detailed understanding of maternal workload and childcare practices at the time of exclusive breastfeeding cessation could provide more context for this finding. EBF duration was not significantly associated with household socioeconomic position (SEP). However, future work should carefully consider sociocultural influences on infant feeding practices in this agropastoral population, traditional structure of asset ownership (e.g., livestock are owned predominantly by men, low rates of formal education), and the complex interplay between these factors and household family structure. Additional demographic and durable asset information in future studies may improve the accuracy of the SEP calculation.

In Chapter 3, I reported on the results of mid-infrared technology to measure milk macronutrient concentrations (fat, total protein, lactose, TRP), and mass spectrometry to quantify the relative abundances of HMO classes (sialylated, fucosylated, undecorated, sia-fuc) and HMGPs (lactoferrin, IgA) in samples collected from a subset of the HERO-G subsample across the first year of lactation. Milk composition (concentrations of macronutrients and relative abundances of HMOs and HMGPs) aligned with results from other studies quantifying macronutrients and previous studies in this region quantifying HMOs and HMGPs. Seasonality was an important driver of maternal milk

TRP and protein content in PLS models, which may reflect seasonal pressures on immune function, as TRP and protein are macronutrients which encompass many individual immune proteins. Future investigations including detailed information about maternal agricultural workload (including details on specific agricultural tasks, time spent in the field, etc.), and maternal diet/nutritional status at the time of milk production, could provide more context for interpreting these findings. Collection time point, representative of stage of lactation, was a significant predictor of milk composition, likely due to temporally-driven pressures on milk constituents and shifts in infant immunological/nutritional needs as complementary foods are being introduced.

In Chapter 4, I assessed and described infant morbidity occurrences across the first 12 months of life in the HERO-G subsample using data recorded by clinicians at the MRC Keneba field station at scheduled and non-scheduled visits (caregivers sought and were provided MRC clinical evaluation and treatment for infant morbidity as needed). By 12 months of age, infants exclusively breastfed to 6 months or longer had significantly fewer morbidity reports relative to those who began receiving non-breast milk foods before 6 months of age. Mixed model results showed that longer exclusive breastfeeding duration predicted significantly fewer occurrences of infant morbidity across the first year of life, and PLS models show both "real time" and "extended" effects of exclusive breastfeeding duration on morbidity occurrence. This suggests that breastfeeding plays an important role in mediating illness during infancy and childhood in this environment. All of the milk constituents quantified here were influential on morbidity except fat and lactoferrin. Greater total milk protein content and relative abundance of sialylated HMO predicted more morbidity occurrences, and higher concentrations of TRP predicted lower occurrence of morbidity across the first year of life. True protein includes immune proteins, which have established protective effects for infant immune system maturation. Similarly, sialylated and undecorated HMOs have been shown to have protective effects and capabilities to treat chronic inflammation; the positive association might represent maternal milk synthesis responding to infant needs. The negative relationships between morbidity and abundance of fucosylated and sia-fuc HMOs aligns with research from other studies. Future research should conduct a more thorough investigation of the milk proteome and individual HMO structures in relationship to infant outcomes in this population.

In Chapter 5, I compiled results from Chapters 2-4 and assessed their relationships to infant growth outcomes, with a focus on WHZ, HAZ, and WAZ across the first 12 months of life. Growth outcomes followed patterns documented in rural Gambia over the last four decades, where, at birth, infants are small, and they continue to fall away from the WHO child growth centiles over the first two years of life[261]. Diet and illness are factors that are known

to influence infant growth outcomes in a complex manner. Morbidity and milk composition had strong impacts on growth outcomes in the statistical models. Using symptomatic infant morbidity occurrence as a proxy for immune activation, these results likely indicate that chronic or more frequent symptomatic illnesses divert resources away from growth and towards physiological systems for self-maintenance (immune defense). This may be a strategy to increase survivorship and maximize fitness under harsh environmental conditions. Future investigations may consider assessing milk micronutrients, gut microbiota composition, biological markers of inflammation, exposure or risk of exposure to pathogens, and more detailed assessments of household SEP, all of which have been shown to impact growth or physiological systems related to growth in existing studies. Finally, infant fecal pH was linked to certain growth outcomes in this population, and a larger sample size will be helpful in identifying any population-level patterns. This work sets a strong foundation for such future investigations.

CONCLUSIONS

In this project, I set out to investigate the impact of first foods on infant outcomes in a low-income population from the rural West Kiang region of The Gambia. Using longitudinal data collected as a part of the larger HERO-G project, I found evidence to suggest that exclusive breastfeeding and maternal milk macronutrients, HMOs, and HMGPs mediate morbidity during the first year of life in this environment. Greater morbidity occurrence negatively influenced infant weight-for-height and weight-for-age across the first year of life, with both "real time" and "extended" effects, suggesting a possible tradeoff between immune function and growth (particularly body weight) in this population. Similar findings have been noted in previous studies from this region.

Research on maternal milk composition and infant health and growth outcomes continues to expand. Still, there are knowledge gaps left to bridge. For example, maternal Secretor status (as designated by relative abundances of a1-2 fucosylated HMOs) was inconsistent across the course of lactation for some mothers. This raises questions as to the accuracy of using milk phenotypes in place of genotyping. A lack of uniformity across lactation may indicate a greater degree of plasticity in milk composition than previously understood. Future analyses investigating possible alternative pathways that may influence milk composition (including functional mechanisms related to Secretor status) are required to unpack this finding.

Additionally, revisiting details of household socioeconomic position, especially as it relates to maternal agricultural employment in the contemporary setting throughout pregnancy and over the course of lactation in the

West Kiang region – a topic that has been rigorously investigated in earlier studies – would provide further insight into the biological influences of the livelihoods of the communities in this region. Interviews or focus groups, to supplement questionnaires, might enhance the ability of future studies to provide additional context for the interpretation of the causes and consequences of infant feeding decisions in a highly seasonal environment.

Evaluating drivers of variation in maternal milk composition is critical in understanding how shifts in environment or physiology may contribute to the composition of early life dietary intake and subsequent infant health and growth outcomes. The physiological and nutritional cues received by offspring during early life can significantly impact both short- and long-term metabolic and immune defense function. Morbidity events such as infections, chronic inflammation, and intestinal permeability can impact an infant's ability to utilize nutrients from their dietary intake, which can subsequently impact weight and height. Suboptimal diet during early life can contribute to diminished innate and acquired mucosal defenses, which can subsequently increase an individual's vulnerability to later-life morbidities. Understanding the nuanced changes in milk composition would allow for greater understanding of the relationships between environmental conditions, as experienced by the mother, and their impact on infant diet. Without measurements of milk volume intake, however, precise claims regarding the amount of nutritional and immunological factors received from mother's milk are not feasible.

Few longitudinal studies have comprehensively evaluated the interrelationships between milk composition, infant feeding practices, and infant health and growth outcomes. Examining these variables in isolation, or as part of a cross-sectional study design, may impact a researcher's ability to discriminate between individual and combined effects on outcomes of interest. A comprehensive understanding of the influence of early life diet on health and growth outcomes is of particular importance in low-income populations that experience marked seasonality associated with annual food insecurity, heavy maternal workload, and fluctuations in infectious disease burden. In such populations, these factors influence early life growth outcomes and are often linked to high rates of infant and childhood morbidity and mortality.

In addition to building a longitudinal framework for understanding the effects of first foods on infant health and growth outcomes, this study lays the groundwork for future evaluations of fecal pH as a reliable proxy for breastfed infant intestinal ecology and gut microbiota composition. This is study is among the first to evaluate relationships between fecal pH and infant health and growth outcomes across the first year of life in a population under significant

environmental and physiological stress. Future work will look in detail at how fecal pH relates to gut microbiota composition in HERO-G infants.

Early life diet is a complex multi-faceted system. In this dissertation, I found that maternal milk composition is influenced by a number of exogenous factors, and that breastfeeding practices and maternal milk composition are important drivers of infant health and growth outcomes. I found evidence of extended effects of early life diet on health and growth outcomes in this cohort, highlighting that first foods can make persistent contributions and leave lasting impacts on offspring outcomes. Future research is needed to explore other physiological, evolutionary, ecological, and sociocultural mechanisms that help address questions related to nutrition, variation in human milk profiles, and subsequent influences on infant health and growth outcomes.

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Appendix

Table A.1 HERO-G maternal milk collection protocol

(1) Upon arrival at the clinic, mothers should be instructed to nurse infants from the right breast. Thereafter, mothers should not nurse their infants from that breast until the time of milk collection. Mothers can feed from their left breast until that time.

(2) Breast milk samples should be collected at approximately 1:00 pm (13:00). Mothers should eat lunch before breast milk collection commences, and during collection mothers should be seated in a comfortable location. A female field or clinic worker should be present during milk collection, to assist with the infant while the mother hand-expresses her milk.

(3) Once the mother is seated comfortably, she should be instructed to nurse her infant from her right breast for 2-3 minutes. After 2-3 minutes, the infant should be gently removed from the breast and handed to the female field or clinic worker.

(4) The mother should then hand-express 10mL of milk into 2 pre-labelled 5mL universal tubes, each filled to the 5mL level. Tubes will be labelled with the infant's study ID (e.g. CHG0001A) and the study visit and breast will be indicated, e.g. WK12LB, WK12RB, WK24LB, WK24RB. Tubes should be placed in an ice bucket once filled.

(5) Time since last feed (time of nursing from right breast upon arrival at clinic) and time of collection must be recorded.

(6) After collection, breast milk samples should be gently inverted (not vortexed) and separated into 6 smaller aliquots for storage (4 x 2mL and 2 x 1mL). Where less than 10mL milk is collected, 2mL aliquots should be prepared first, and 1mL aliquots prepared from the remainder as available.

Table A.2. Maternal MUAC (Milk analysis subset N=50)

Time Point	*N*	*M*	*SD*
20wks	*24*	*26.55*	*2.06*
28wks	*50*	*26.19*	*2.29*
36wks	*49*	*26.25*	*2.29*
Delta20-36	*24*	*0.35*	*1.68*
Delta28-36	*49*	*0.09*	*1.28*

Table A.3. Maternal MUAC (HERO-G subsample N=194)

Time Point	*N*	*M*	*SD*
20wks	*94*	*27.24*	*2.83*
28wks	*193*	*26.94*	*2.35*
36wks	*189*	*26.78*	*2.15*
Delta20-36	*94*	*0.06*	*1.02*
Delta28-36	*188*	*0.09*	*1.02*

Table A.4. Maternal MUAC (HERO-G cohort N=238)

Time Point	*N*	*M*	*SD*
20wks	*118*	*27.07*	*3.8*
28wks	*235*	*26.84*	*3.43*
36wks	*228*	*26.78*	*3.18*
Delta20-36	*116*	*0.09*	*1.02*
Delta28-36	*226*	*0.06*	*1.04*

Table A.5. Mean (SD) relative abundances of individual milk HMO structures across the first 12 months post-partum

Time point	3		6		9		12	
HMO	**NS (N=8)**	**S (N=20)**	**NS (N=13)**	**S (N=32)**	**NS (N=10)**	**S (N=36)**	**NS (N=12)**	**S (N=38)**
LNT	31.94 (10.5)	26.8 (6.3)	35.02 (13.5)	23.56 (5.6)	40.25 (15.1)	26.48 (7.7)	36.88 (14.3)	0.36 (0.3)
LNFP II	13.14 (4.0)	8.27 (4.0)	13.55 (4.3)	10.40 (3.4)	13.01 (4.8)	9.55 (4.3)	13.04 (5.3)	11.53 (7.8)
3FL	11.75 (1.1)	6.37 (3.7)	15.83 (5.8)	10.01 (4.5)	14.75 (4.6)	9.65 (5.2)	17.95 (5.1)	11.71 (6.4)
DFLNHb	4.14 (1.9)	1.36 (1.3)	3.64 (1.6)	1.87 (1.1)	3.65 (2.3)	1.63 (1.1)	3.08 (1.6)	1.48 (1.0)
MFLNH I + III	3.44 (1.9)	1.09 (1.2)	2.75 (2.2)	1.02 (0.9)	2.60 (2.4)	0.93 (0.9)	2.15 (2.1)	0.74 (0.7)
LSTb	2.94 (1.1)	1.76 (0.57)	2.36 (0.8)	1.43 (0.5)	2.13 (0.8)	1.56 (0.7)	2.30 (0.7)	9.61 (4.6)
2020a	2.44 (0.4)	1.21 (0.8)	3.28 (1.5)	2.01 (1.1)	3.01 (1.4)	1.91 (1.2)	3.75 (1.4)	2.36 (1.5)
4210a	2.39 (0.9)	1.74 (0.8)	1.41 (0.7)	1.64 (0.8)	1.50 (0.6)	1.40 (0.8)	1.17 (0.7)	1.13 (0.9)
LNDFH II	2.30 (1.5)	0.97 (0.7)	2.14 (1.8)	1.16 (0.8)	1.78 (1.8)	1.07 (0.8)	2.21 (2.0)	0.27 (0.2)
LNFP V	2.03 (0.3)	0.61 (0.3)	2.23 (0.8)	0.75 (0.3)	2.27 (0.6)	0.75 (0.4)	2.84 (0.7)	0.15 (0.2)
LNDFH I	1.98 (0.7)	4.90 (2.3)	2.25 (1.2)	5.10 (2.4)	2.12 (1.2)	4.6 (2.7)	2.30 (1.3)	1.19 (0.9)
FS-LNnH I	1.84 (0.9)	0.99 (1.0)	1.20 (0.6)	0..99 (0.8)	0.76 (0.4)	0.67 (0.7)	0.55 (0.3)	0.41 (0.4)
LNFP III + LNFP I	1.55 (1.2)	11.67 (5.7)	1.19 (1.2)	10.55 (6.1)	0.94 (0.9)	11.79 (6.8)	1.16 (0.9)	0.88 (0.4)
IFLNH III	1.32 (0.7)	0.83 (0.6)	0.77 (0.6)	0.87 (0.4)	0.59 (0.4)	0.73 (0.5)	0.5 (0.4)	0.54 (0.4)
LSTc	1.10 (0.8)	1.64 (0.8)	0.63 (0.5)	0.84 (0.5)	0.42 (0.3)	0.62 (0.4)	0.28 (0.2)	1.47 (0.6)
3000a	1.05 (0.3)	0.91 (0.2)	1.55 (0.4)	1.14 (0.26)	1.61 (0.4)	1.16 (0.2)	1.69 (0.6)	0.17 (0.3)
3000c	0.97 (0.5)	0.49 (0.5)	0.85 (0.4)	0.50 (0.3)	0.66 (0.3)	0.46 (0.3)	0.73 (0.5)	0.14 (0.1)
6'SL	0.96 (0.4)	0.88 (0.4)	0.68 (0.4)	0.55 (0.3)	0.46 (0.3)	0.43 (0.3)	0.27 (0.2)	0.30 (0.2)
MFpLNH IV	0.95 (0.3)	0.33 (0.3)	0.89 (0.3)	0.49 (0.3)	0.89 (0.5)	0.43 (0.3)	0.76 (0.3)	0.40 (0.3)
3200b	0.92 (0.5)	1.30 (0.6)	0.47 (0.4)	0.64 (0.6)	0.33 (0.2)	0.61 (0.5)	0.33 (0.3)	0.41 (0.4)
S-LNnH II	0.83 (0.6)	1.70 (1.1)	0.34 (0.2)	0.68 (0.6)	0.21 (0.2)	0.54 (0.5)	0.16 (0.1)	0.29 (0.3)
LNnH	0.80 (0.7)	3.55 (2.5)	0.41 (0.5)	2.15 (2.9)	0.34 (0.3)	2.17 (2.8)	0.24 (0.3)	2.07 (2.9)
LNH	0.78 (0.5)	1.18 (0.6)	0.34 (0.3)	0.53 (0.5)	0.24 (0.1)	0.50 (0.5)	0.24 (0.2)	0.33 (0.4)
DFpLNH II	0.72 (0.5)	0.29 (0.3)	0.42 (0.4)	0.29 (0.3)	0.35 (0.4)	0.27 (0.3)	0.40 (0.4)	0.23 (0.3)
4110a	0.60 (0.3)	0.17 (0.1)	0.84 (0.3)	0.31 (0.2)	0.87 (0.3)	0.30 (0.2)	0.93 (0.4)	0.31 (0.2)
3110c	0.37 (0.2)	0.14 (0.1)	0.20 (0.1)	0.12 (0.1)	0.17 (0.1)	0.13 (0.1)	0.19 (0.1)	0.06 (0.05)
F-LSTc	0.35 (0.3)	0.21 (0.5)	0.18 (0.2)	0.19 (0.2)	0.11 (0.1)	0.12 (0.1)	0.09 (0.1)	4.69 (2.9)
5310b	0.33 (0.2)	0.14 (0.1)	0.22 (0.3)	0.09 (0.1)	0.21 (0.3)	0.09 (0.1)	0.15 (0.2)	0.06 (0.1)
2020b	0.33 (0.1)	1.06 (0.3)	0.27 (0.1)	1.13 (0.3)	0.18 (0.1)	1.11 (0.3)	0.18 (0.1)	1.16 (0.3)
3'SL	0.29 (0.1)	0.32 (0.1)	0.53 (0.4)	0.37 (0.2)	0.43 (0.1)	0.41 (0.2)	0.49 (0.2)	0.45 (0.2)
2100b	0.29 (0.1)	0.2 (0.1)	0.17 (0.1)	0.14 (0.05)	0.17 (0.1)	0.15 (0.1)	0.16 (0.1)	0.14 (0.1)
2'FL	0.28 (0.1)	7.52 (2.5)	0.37 (0.3)	8.72 (2.5)	0.21 (0.2)	8.93 (2.6)	0.25 (0.2)	9.67 (2.9)
DFLNO I	0.28 (0.1)	0.11 (0.1)	0.15 (0.1)	0.08 (0.06)	0.14 (0.08)	0.07 (0.05)	0.10 (0.1)	0.04 (0.04)
4220a	0.27 (0.1)	0.05 (0.1)	0.30 (0.2)	0.06 (0.06)	0.30 (0.2)	0.05 (0.04)	0.23 (0.1)	0.05 (0.1)
p-LNH	0.26 (0.2)	0.86 (0.7)	0.18 (0.1)	0.43 (0.5)	0.18 (0.1)	0.55 (0.6)	0.15 (0.1)	0.38 (0.5)
5310c	0.26 (0.2)	0.24 (0.1)	0.10 (0.1)	0.16 (0.1)	0.09 (0.1)	0.13 (0.1)	0.07 (0.1)	0.09 (0.1)
DFLNnO II	0.25 (0.2)	0.18 (0.2)	0.14 (0.2)	0.12 (0.1)	0.11 (0.1)	0.10 (0.1)	0.10 (0.1)	0.07 (0.1)
2010b	0.25 (0.1)	0.13 (0.04)	0.17 (0.2)	0.14 (0.1)	0.14 (0.2)	0.15 (0.1)	0.21 (0.2)	0.17 (0.1)
6410a	0.21 (0.2)	0.10 (0.1)	0.05 (0.05)	0.05 (0.06)	0.05 (0.05)	0.04 (0.1)	0.03 (0.05)	0.02 (0.1)
3110d	0.20 (0.1)	0.12 (0.1)	0.15 (0.1)	0.12 (0.1)	0.10 (0.04)	0.09 (0.1)	0.08 (0.03)	0.09 (0.1)
FS-LNO	0.19 (0.1)	0.14 (0.1)	0.09 (0.1)	0.08 (0.04)	0.07 (0.05)	0.06 (0.04)	0.05 (0.04)	0.03 (0.03)
3110b	0.18 (0.1)	0.15 (0.1)	0.11 (0.1)	0.19 (0.2)	0.09 (0.1)	0.18 (0.2)	0.09 (0.1)	0.11 (0.1)
4210a	0.17 (0.1)	0.23 (0.2)	0.09 (0.1)	0.16 (0.1)	0.07 (0.1)	0.15 (0.1)	0.08 (0.1)	0.12 (0.1)
6400a	0.16 (0.2)	0.23 (0.1)	0.07 (0.1)	11.74 (0.1)	0.05 (0.06)	0.10 (0.1)	0.03 (0.1)	0.06 (0.1)
4230a	0.16 (0.1)	0.11 (0.1)	0.10 (0.10)	0.10 (0.1)	0.08 (0.1)	0.08 (0.1)	0.10 (0.1)	0.07 (0.1)
3000d	0.15 (0.1)	0.15 (0.2)	0.16 (0.1)	0.14 (0.1)	0.18 (0.1)	0.15 (0.1)	0.18 (0.2)	0.02 (0.05)
3120a	0.15 (0.1)	0.34 (0.3)	0.10 (0.1)	0.42 (0.2)	0.09 (0.1)	0.35 (0.2)	0.10 (0.1)	0.15 (0.1)
3000b	0.14 (0.1)	0.12 (0.1)	0.15 (0.06)	0.14 (0.22)	0.14 (0.03)	0.11 (0.04)	0.15 (0.04)	0.42 (0.2)
4100a	0.14 (0.1)	0.27 (0.2)	0.12 (0.1)	0.21 (0.2)	0.11 (0.05)	0.22 (0.2)	0.11 (0.04)	0.22 (0.2)
5320a	0.14 (0.1)	0.19 (0.1)	0.08 (0.04)	0.13 (0.06)	0.07 (0.06)	0.11 (0.1)	0.06 (0.04)	0.07 (0.1)
6410a	0.14 (0.1)	0.11 (0.1)	0.13 (0.2)	0.08 (0.1)	0.13 (0.2)	0.07 (0.1)	0.09 (0.2)	0.04 (0.1)
4210b	0.14 (0.03)	0.05 (0.03)	0.09 (0.04)	0.05 (0.03)	0.08 (0.04)	0.05 (0.03)	0.06 (0.03)	0.04 (0.02)
5300a	0.12 (0.1)	0.47 (0.3)	0.06 (0.07)	0.25 (0.3)	0.05 (0.05)	0.23 (0.3)	0.04 (0.05)	0.18 (0.3)
4100b	0.10 (0.1)	0.06 (0.03)	0.06 (0.04)	0.04 (0.02)	0.05 (0.03)	0.04 (0.03)	0.05 (0.03)	0.03 (0.02)
DFLNHa	0.10 (0.1)	0.59 (0.3)	0.07 (0.04)	0.73 (0.9)	0.05 (0.04)	0.64 (0.9)	0.05 (0.04)	0.52 (0.7)
3120b	0.08 (0.1)	0.14 (0.1)	0.05 (0.04)	0.15 (0.05)	0.04 (0.05)	0.15 (0.1)	0.05 (0.05)	0.10 (0.1)
5310a	0.07 (0.1)	0.06 (0.1)	0.03 (0.02)	0.04 (0.02)	0.03 (0.02)	0.03 (0.03)	0.02 (0.02)	0.02 (0.02)
TFLNH	0.07 (0.04)	0.20 (0.2)	0.05 (0.04)	0.16 (0.2)	0.05 (0.05)	0.12 (0.1)	0.04 (0.03)	0.10 (0.1)
2010c	0.06 (0.03)	0.26 (0.1)	0.05 (0.04)	0.38 (0.2)	0.04 (0.04)	0.43 (0.2)	0.07 (0.1)	0.49 (0.2)
2010a	0.06 (0.02)	0.23 (0.1)	0.05 (0.04)	0.36 (0.2)	0.04 (0.04)	0.41 (0.2)	0.07 (0.1)	0.47 (0.2)
5301a	0.05 (0.03)	0.10 (0.1)	0.02 (0.01)	0.04 (0.03)	0.01 (0.01)	0.03 (0.02)	0.01 (0.01)	0.01 (0.01)
2100a	0.04 (0.02)	0.03 (0.02)	0.04 (0.02)	0.03 (0.02)	0.06 (0.02)	0.04 (0.02)	0.05 (0.02)	0.05 (0.02)
DFS-LNH	0.03 (0.02)	0.08 (0.1)	0.02 (0.02)	0.03 (0.03)	0.01 (0.02)	0.02 (0.02)	0.01 (0.01)	0.01 (0.01)

Time point	**3**		**6**		**9**		**12**	
4220b	0.03 (0.01)	0.18 (0.1)	0.01 (0.01)	0.11 (0.1)	0.01 (0.01)	0.10 (0.1)	0.01 (0.01)	0.07 (0.1)
3000e	0.02 (0.01)	0.03 (0.1)	0.02 (0.02)	0.03 (0.1)	0.01 (0.01)	0.01 (0.02)	0.01 (0.01)	25.37 (8.1)
3200a	0.02 (0.01)	0.35 (0.3)	0.02 (0.02)	0.13 (0.1)	0.02 (0.02)	0.16 (0.2)	0.01 (0.01)	0.05 (0.02)
IFLNH I	0.01 (0.02)	0.25 (0.3)	0.01 (0.01)	0.11 (0.1)	0.01 (0.01)	0.18 (0.2)	0.01 (0.01)	0.12 (0.2)
5300b	0.01 (0.01)	0.08 (0.1)	0.01 (0.01)	0.04 (0.1)	0.005 (0.004)	0.04 (0.1)	0.004 (0.01)	0.03 (0.1)
DFS-LNHnH	0.01 (0.004)	0.08 (0.1)	0.004 (0.002)	0.07 (0.06)	0.003 (0.002)	0.04 (0.1)	0.003 (0.002)	0.02 (0.02)
LDFT	0.01 (0.002)	2.14 (1.7)	0.01 (0.01)	3.98 (2.8)	0.01 (0.01)	3.28 (2.5)	0.01 (0.01)	5.01 (3.0)
4110b	0.002 (0.001)	0.11 (0.1)	0.002 (0.003)	0.07 (0.1)	0.002 (0.002)	0.08 (0.1)	0.002 (0.002)	0.07 (0.1)

Table A.6. Infant morbidity categories and specific diagnoses.

Dermatological	Ear	GI	Hematological	Nutritional	Ophthalmological	Oral	Other Infectious	Respiratory	Urinary
Allergic urticaria	Chronic suppurative otitis media	Bacterial intestinal infection	Anemia	Hypoglycemia	Conjunctivitis	Aphthous Ulcers	Herpesviral infection, unspecified	Asthma	Urinary tract infection
Cellulitis	Nonsuppurative Otitis Media	Constipation	Bacterial sepsis	Marasmus	Hyphaema	Candidiasis	Lymphadenitis	Bronchiolitis	
Cheilitis	Otitis externa	Giardia	Septicemia	Severe protein-energy malnutrition		Gingivitis	Plasmodium falciparum malaria	Chronic bronchitis	
Cold sore	Suppurative otitis media	Hookworm disease		Vitamin C deficiency		Glossitis	Typhoid fever	Common Cold	
Cutaneous abscess, furuncle & carbuncle		Intestinal helminthiasis				Oral candidiasis		Croup	
Eczema		Non infective diarrhea				Peritonsillar abscess		Pneumonia	
Impetigo		Peptic Ulcer Disease				Stomatitis			
Skin/subcutaneous tissue infection		Viral Gastroenteritis				Tonsillitis			
Paronychia									
Post injury infected wound									
Ring Worm									
Scabies									
Seborrheic dermatitis									
Unspecified contact dermatitis									
Unspecified Rash									
Urticaria									
Varicella									

*Morbidity classifications include: Respiratory (pneumonia, common cold, bronchiolitis, asthma); Dermatological (eczema, contact dermatitis, impetigo, allergic urticaria, rashes, cheilitis, cold sores, abscesses, paronychia, seborrheic dermatitis, ring worm, scabies, local infection of skin); Gastrointestinal (viral gastroenteritis, intestinal helminthiasis, bacterial intestinal infection, non-infective diarrhea, hookworm disease, giardia, peptic ulcer disease, constipation); Ophthalmological (conjunctivitis, hyphaema); and Oral (oral candidiasis, tonsillitis, glossitis, stomatitis, peritonsillar abscess, gingivitis, aphthous ulcers).

Table A.7. Cumulative morbidity at 3, 6, 9 and 12mo of life according to sex, birth season, parity, and EBF duration (milk analysis subset)

Age (mo)	Value	Female (N=28)	Male (N=22)	Dry (N=39)	Wet (N=11)	Multiparous (N=48)	Primiparous (N=2)	EBF <6mo (N=36)	EBF ≥6mo (N=14)
3	Mean	3.2	3.73	3.11	**4.5***	3.35	6	**4.12***	1.7
	SD	2.0	3.13	2.63	1.77	2.51	.	2.60	1.06
	N (infants)	20	15	27	8	34	1	25	10
	Sum (morbidity)	64	56	84	36	114	6	103	17
6	Mean	4.33	5	4.37	5.75	4.55	8	**5.16***	3.25
	SD	2.32	3.74	3.14	2.19	3.0	.	3.29	1.48
	N (infants)	24	19	35	8	42	1	31	12
	Sum (morbidity)	104	95	153	46	191	8	160	39
9	Mean	6.32	7.29	6.52	7.6	6.67	11	7.25	5.64
	SD	3.22	5.68	4.58	4.25	4.49	.	4.82	3.52
	N (infants)	25	21	36	10	45	1	32	14
	Sum (morbidity)	158	153	235	76	300	11	232	79
12	Mean	7.65	8.86	7.59	10.27	8.24	7.5	8.76	6.86
	SD	4.19	6.08	4.91	5.47	5.05	9.19	5.61	3.46
	N (infants)	26	22	37	11	46	2	34	14
	Sum (morbidity)	199	195	281	113	379	15	298	96

*P<0.05; **P<0.01; A variable with a significant difference (indicated by boldface font and p-value<0.05 or 0.01) indicates a significantly *greater* number of morbidity occurrences relative to its comparative group (e.g., a boldface p-value in the EBF <6mo column indicates that infants EBF <6mo experienced significantly more morbidity occurrences compared to infants EBF ≥6mo).