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The Developmental Ecology of the Infant Gut Microbiome

By

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Doctor of Philosophy

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B.A., Sarah Lawrence College, 2002
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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Abstract

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By Gabriela M. Sheets

Introduction: Within the human intestinal tract lives a complex and dynamic community of microorganisms, called the intestinal microbiome. Human behavior and ecology play central roles in shaping this resident community during development. The microbiome assembles anew with each host generation, and given its critical role in human somatic, immune and metabolic development, natural selection likely has conserved mechanisms for the intergenerational transfer such that infants receive an optimal supply of human-adapted microbiota. Research shows that contemporary birthing methods and feeding practices disrupt the successional inheritance during early life, yet little is known about how diverse developmental ecologies influence the infant gut microbiome.

This dissertation is a biocultural, multi-disciplinary, and longitudinal exploration of microbial development within a semi-rural Salvadoran population. Assuming a developmental ecology framework, I probe the broader socio-political and economic processes acting upon infant microbial ontogeny via proximal developmental ecologies that mediate exposure. I test two propositions: First, the ontogeny of the microbiome requires an initial vertically selected microbiota, and with the continued protection of breastmilk, it increasingly demands a more diverse, horizontally-transmitted microbial assortment. Second, contemporary behaviors can interrupt the timeline of vertical and horizontal exposures, resulting in altered microbial assembly, growth and health outcomes.

Methods: 71 caretaker-infant pairs were recruited and followed for 12 months. Three study phases included collection of interviews, 24-hour dietary recalls, health histories, anthropometrics, participant observation, and fecal samples. Fecal samples were frozen (-20°C) and transported to CU Boulder for 16S rRNA sequencing.

Results: Factors influencing the vertical transmission (birth-mode and early feeding), and those influencing horizontal transmission (childcare networks, gendered labor patterns, and household microbial ecologies) significantly affected infant microbial diversity, stability and composition in the first year. Altered colonization patterns were associated with distinct growth phenotypes and health outcomes.

Discussion: Through comparative analyses and the development of a tri-fold, microbe-host-ecology integrative model, I explored the timeline of vertical and horizontal exposures, identified ontological microbial variations, and assessed functional relationships with health and growth outcomes. Novel analytic methods were developed to identify intra-population parameters of age-dependent “healthy” microbiome development. Recommendations for microbial interventions and policy-makers were made to support a holistic and life-course view of humans and their microbial partners.

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Whenever I think of my experience at Emory, I think of George Armelagos. A life-long friend and hero, I am forever grateful for the opportunity to teach, publish, and dine with him. My gratitude to the staff of the Department of Anthropology at Emory, particularly Lora McDonald for creating a stable, thriving and student-friendly graduate studies program, and to Jill Marshall for building upon this foundation. In addition to my dissertation committee, thanks for all of the intellectual support from Peter Brown, Paul Hooper, and David Nugent. Thanks to Tess Pendergrast, Isabella Alexander, Hilary King, Tawni Tidwell, Jennifer Sarrett, and Melissa Creary for the friendship, edits and intellectual stimulation. Thanks to Luis Zuleta Benavides for his un-ending patience and support with excel spreadsheets, dreaded R code and brightening my world with a desk-top monitor. I can’t express enough gratitude for Dr. Cesar and Brian Nguyen for bringing endless joy and health back into my life.

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The following section is the most sensitive and difficult part of this dissertation for me to write. On the evening of February 24th, 2016, my collaborator, friend and the public health promoter, Ana Carmen Díaz de Marroquín together with her husband Santos, were killed in their living room. Five young gang members slipped into Ana's house on this unsuspecting evening, tortured and shot the couple in front of their three grandchildren. Purportedly the act was retaliation for Ana's meeting with the national police regarding a prior gang-related murder. Her murder was meant to send a message to the village. That message was certainly received.

Niña Ana's relentless commitment and love for her community earned her a deep respect and a saintly prestige. Her human form failed her in that she could not appear in all places at once: daily, Ana visited and cared for elderly, conducted perinatal visits, weighed, vaccinated and cared for every infant under 24 months, located and eradicated dengue sources, provided women with contraceptive and other health services, recorded and reported deaths, and performed autopsies among other key tasks. Walking down the street with Ana was a dynamic event, as people of all ages rushed into the street to ask her for assistance or advice. She was tireless and often stayed up late at night or woke early in the morning to scratch out her impeccable and extensive paper records regarding the 5,000 inhabitants she was responsible for. Niña Ana was NGO-trained in the 1980s, and her dedication and hard work kept a splintered community together.

I do not exaggerate when I state that this dissertation would not have been possible without Niña Ana. Trust in her opened nearly every door in Joya de Cerén to me and my research team. She helped me to recruit and retain study families in a village where the only constant is environmental, political economic and social instability. Niña Ana painstakingly guided me through dangerous situations, which allowed me to continue my research through otherwise untenable circumstances, and likely saved my life more than once.

No matter how one understands the historical context of CIA involvement and US gang extradition, nor the compassion one conjures for young Salvadoran boys with no hope and little future, no matter anything, her loss is both immense and immeasurable. In the year since her death she has not been replaced, and Joya de Cerén languishes in her absence. The sliver of comfort I find, is remembering how Ana took joy in and believed in this study. Recruiting mothers, she always said "Imagine all of the babies that you'll be helping in the future!"

Niña Ana, this is for you, and for a brighter future for El Salvador. You live on through all the many lives you have touched, including my own, and those that I hope to have the privilege of touching in the future.

Dedicado a Niña Ana Carmen Díaz de Marroquín (1957-2016).

Gracias por su arduo trabajo, servicio y amistad. Esta investigación no habría sido posible sin Ud. Espero que esté orgullosa de este trabajo.

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Social and Ecological Indexes

HMEI: Household Microbial Ecology Index (Ch. 3-Ch.5)

- Low HMEI=high levels of microbial exposure
- Middle HMEI = mid-levels of microbial exposure
- High HMEI = low levels of microbial exposure

SES: Socioeconomic status (Ch. 3-Ch.5)

- Low SES
- Middle SES
- High SES

Early feeding

BF: Breastfed
MF: Mixed-fed
FF: Formula-fed

Birth mode

VD: Vaginally delivered
CD: Cesarean delivered

Sub groups of early feeding and birth mode

VDBF: Vaginally delivered, breastfed
CDBF: Cesarean delivered, breastfed
VDFF: Vaginally delivered, breastfed
CDFF: Cesarean delivered, formula fed

Growth

UGP: Underweight growth phenotype

AGP: Average growth phenotype

OGP: Overweight growth phenotype

Microbial metrics

ADR: Ideal age-dependent range for bacterial colonization

ADR-a: Ideal age-dependent range for alpha diversity

Terms

A-diversity: Alpha diversity is a summary measure of intra-sample taxonomic diversity, which captures both the organismal richness of the sample and the evenness of the organisms' abundance distribution.

Dysbiosis: Imbalance of a microbial community

Human milk olisaccharides (HMOs): are complex sugars that play key roles in driving the community composition and diversity of the infant gut microbiota (Sela and Mills 2010).

Microbial Ecology: describes the material environment(s) where microbes are transmitted to host through contact.

Pathobiont: members of the commensal microbiota that have the capacity to behave as pathogen. A term that attempts to capture the complexity of microbiome, microbiome-host, and microbiome-host-ecology interactions. Unfortunately, it reduces these complex context and age-dependent variables to a "good and bad" status without explaining which variables make it such, but there also is some value in a quick label that allows readers to flag this complex interaction.

CHAPTER 1: INTRODUCTION

Within the human intestinal tract lives a complex and dynamic community of microbes, called the intestinal microbiome. Human behavior and ecology play central roles in shaping this resident community, and consequently, microbiome composition exhibits wide variability among and within individuals. Although these dynamics are manifested first in ontogeny, they can have lasting effects on health and well-being throughout the life course. Intestinal microbiota have been shown to play roles in key biological systems, including metabolic function (Ley et al. 2006a; Turnbaugh et al. 2006; Blanton et al. 2016), immune regulation (Dillon et al. 2016a), pathogen defense (Cho and Blaser 2012; Honda and Littman 2012), and cognition (mood, neuroregulation) (Galland 2014).

Extensive host-microbe coevolution informs these roles. Humans and other terrestrial vertebrates share related, yet distinct microbial lineages (Gong et al. 2007; Mackie et al. 2004; Nelson et al. 2003; Uenishi et al. 2007; Wilson et al. 2006), indicating that over time, bacterial taxa have adapted to their human hosts (Cullen et al. 2015; Kwong et al. 2014). In turn, humans have adapted to their resident microbiota in three ways: (1) physiologically (Charbonneau et al. 2016), (2) immunologically (Stappenbeck et al. 2002; Chung et al. 2012) and (likely) (3) behaviorally (Cryan and O'Mahony 2011; Neufeld et al. 2011). This evolutionary process has been three-fold: involving interactive feedback loops between humans, their resident microbes, and changing environments, making the acquisition of an ecologically and host-tailored microbiota critical. Yet contemporary birthing methods and feeding practices have been shown to disrupt the successional inheritance during early life (Dominguez-Bello et al. 2010; Thompson 2012). As the bourgeoning field of microbiomics endeavors to translate these rapidly expanding insights into health care policy and intervention, research incorporating theoretical frameworks that account for a tri-fold developmental system are increasingly important.

This dissertation is a biocultural and interdisciplinary exploration of early microbiome development within a semi-rural Salvadoran population. To fully explore the tri-fold, microbial development of this population, these chapters necessarily weave through multiple levels of analysis, from broader socio-political processes to household dynamics, into infant gut microbiota, and back out through infant growth parameters and health outcomes, ultimately to develop policy implications. To accomplish this comprehensive and ambitious task, this dissertation draws from an orientation within human biology called developmental ecology. This theoretical framework views cultural processes as acting upon human biology through their affect on proximal developmental ecologies (Whiting et al. 1975; Worthman 2010). Researchers utilizing this framework show how broader socio-political and economic systems, communities, households and individuals shape proximal developmental "niches" thereby mediating the ways that culture becomes biologically embodied (Munroe et al. 1981; Worthman 2010). Reciprocally, the infant is understood to meet the developmental process with a suite of cognitive, behavioral and physiological dispositions, sensitives, and capacities that had been honed through evolutionary processes (ibid). This dissertation builds on this framework by defining early maternal-infant care practices including birthing and breast-feeding to be a part of this suite of evolved behaviors. In this way, early care practices support the vertical transfer of a co-evolved microbiota, particularly during critical windows of development. The following chapters explore the ways in which these vertical (birth mode, early feeding) and horizontal (socio-ecological processes) microbial transfers are enacted or obstructed, the production of microbial variation, and subsequent effects on infant health and growth. The broadest intention of this dissertation is that through a deep and wide exploration of the tri-fold microbial development of this infant population, tools and methods will emerge to support the operationalization of interventions and policy that can alleviate suffering and enhance well-being in this, and many other populations worldwide.

This introductory chapter is intended to set the stage for a complex and multi-level investigation that makes up the body of the dissertation. The first section of the introduction explores the development of the human gut microbiome, and describes the main hypotheses upon which this dissertation builds. After providing this microbiological and ontological background, a discussion of the integrative research approach, including a political history of El Salvador and the study site Joya de Cerén (pronounced “hoya de seren”), a description of the population and methods is provided. At the foundation of this dissertation is a test of the view that to adequately engage with the microbial ecologies of infants in this Salvadoran village, one must understand the historical-cultural determinants of the setting within which this early development occurs. The final section of the introduction outlines the structure of the dissertation chapters. Ultimately the goal of this dissertation is to help researchers and interventionists broaden their view of early microbiome development to encompass a dynamic three-fold process (human ecology, biology and microbiology), such that effective, locally appropriate interventions can positively affect the health and lives of future generations.

Human-associated microbial development

Vertical colonization patterns

Although the intestinal microbiome assembles anew with each host generation, natural selection likely has conserved mechanisms of transmission (including mammalian biological processes and behaviors) such that offspring receive an optimal supply of human-adapted microbiota. A recent study showed that the microbial gut colonization process may be initiated prenatally by a distinct microbiota in the placenta and amniotic fluid (Collado et al. 2016). The next transfer of key vertically inherited microbiota is seeded as the infant passes through the birth canal. The infant's postnatal microbiota has been shown to be significantly more similar to the mother's vaginal microbiota than to other vaginally born infants, suggesting that the vaginal community is a key factor in the vertical transmission of microbiota from mother to infant (Dominguez-Bello et al. 2010). A growing body of research confirms the prenatal remodeling of the vaginal microbiome in pregnant women. Both overall diversity and richness are reduced over the course of pregnancy, with an increasing dominance of

Lactobacillus species (*L. iners crispatus*, L. *jensenii* and L. *johnsonii*) (Aagaard et al. 2012; Koren et al. 2012). These bacteria are believed to inhibit pathogen growth through secretion of antibacterial bacteriocins as well as the production of metabolites such as lactic acid that help to maintain a low, microbe-hostile pH (Reid et al. 2011). Dysbiosis of the vaginal microbiome is associated with complications in pregnancy, in particular an increased risk of preterm birth (Flynn et al. 1999; Hillier et al. 1995; Donders et al. 2009). Moreover, the treatment of abnormal vaginal microbiota populations with lactobacilli and estriol during pregnancy was found to restore the microbiota to a normal state (Unlü and Donders 2011). Together these findings support the hypothesis that the maternal vaginal microbiome is an important source of the initial bacteria for the neonatal gut microbiome (Jost et al. 2014; Dominguez-Bello et al. 2010; MacIntyre et al. 2015).

Following birth, the infant gut microbiota composition begins to resemble that detected in colostrum and breast milk (Collado et al. 2016). Breastmilk provides nutrients for the maintenance and growth of these nascent microbial communities (Blaut and Clavel 2007). Colostrum and breast milk represent a primary and constant source of maternally selected, commensal and mutualistic bacteria designed to coat the infant gut (Rocío Martín et al. 2003; Heikkilä and Saris 2003; Jiménez et al. 2008; Rocío Martín et al. 2005). A baby consuming approximately 800 mL/day of milk would ingest between 1 × 105 and 1 × 107 bacteria daily (Heikkilä and Saris 2003), explaining why a breastfed infant’s microbiota closely resembles their mother’s breast milk microbiome (Favier et al. 2002). Moreover, human milk olisaccharides (HMOs), or complex sugars, play a key role in driving the community composition and diversity of the infant gut microbiota (Sela and Mills 2010). HMOs are the third-largest solid component of milk and because of their structural complexity they are indigestible to the host, making them a major food source for infant intestinal microbiota (Zivkovic et al. 2011). These complex carbohydrates selectively feed the most abundant bacteria including *Bifidobacterium* and *Lactobacillus*, particularly early in the lactation cycle (Ward et al. 2006). Human milk contains the highest number of distinct oligosaccharides among mammalian milks, comprising close to 200 distinct oligosaccharides (Ninonuevo et al. 2006). Importantly, colostrum contains approximately 22 g/l of oligosaccharides whereas mature milk only contains approximately 10 g/l (Kunz et al. 2000), indicating that the strictest selection of bacteria by means of oligosaccharides occurs in the first months of life, scaffolding selection during the process of vertical microbial inheritance (see Figure 1).

Born with a largely naïve adaptive immune system, infants are at the highest risk of morbidity and mortality in the first months of life. Notably, 44% of child deaths under the age of five take place during the neonatal period (WHO 2015). Infants must therefore, rapidly build immunocompetence—the ability to mount an adequate adaptive immune response—in the first months of infancy. Due to insufficient neonatal production of antigens such as sIgA during the first weeks of life, the high levels of sIgA in the colostrum and early breast milk significantly contribute to the immune protection of an infant. It has been suggested that maternal sIgA may train infant sIgA to accept vertically transmitted microbiota, while rejecting pathogenic and pro-inflammatory bacteria (Hennet and Borsig 2016). Nevertheless, breast milk produces the highest levels of maternal antigens and bioactive immune factors in the first weeks of life. Levels of sIgA reaching 12 g/l are commonly detected in the colostrum, while mature milk contains approximately 1 g/l (Hanson 1961). Thus, the transfer of adaptive secretory immunity from mother to infant provides protection from unwanted microbial invasions while the infant immune system builds competence after the first month of life (Brandtzaeg 2007) (see Figure 1).

Horizontal exposures and colonization patterns

Beginning in the second month of life, infant immune activity rapidly increases. Meanwhile, breast milk composition undergoes a transition: levels of maternal antibodies decrease by more than 90 percent (Hanson 1961) and the diversity of HMOs decrease by 50 percent (Hennet and Borsig 2016), indicating a less specific selection of bacteria. Instead, mature human breast milk increases the number of fat and other nutrients that support infant growth (Hennet and Borsig 2016). Human biologists have hypothesized that during this period of middle-late infancy (4-12 months), the immune system requires cues from the local ecology to support development and regulation (McDade 2005; McDade and Worthman 1999). In light of emerging research that shows the irrevocably interrelated developmental processes of the intestinal microbiome and the immune system (Round and Mazmanian 2009; Thaiss et al. 2016), an increased horizontal exposure during months 2-12 likely becomes critical for the proper regulation and development of both biological systems. This requirement for horizontal exposure “conveniently” emerges at a time when infants gain mobility, increase mouthing behaviors (Fessler and Abrams 2004; Groot et al. 2015; Tulve et al. 2002), and become more independent from their mothers (Sheets and Worthman 2014) (see Figure 1). In this time, infant care networks often increase, care practices evolve, and complementary foods are introduced, all events that likely increase horizontal exposures of infants in the first year of life. This dissertation explores the timeline of exposures and the subsequent variation of bacterial composition and diversity, through comparative analytical methods, in the attempt to define practices and behaviors that encourage healthy development. In doing so, this dissertation builds upon two key hypotheses:

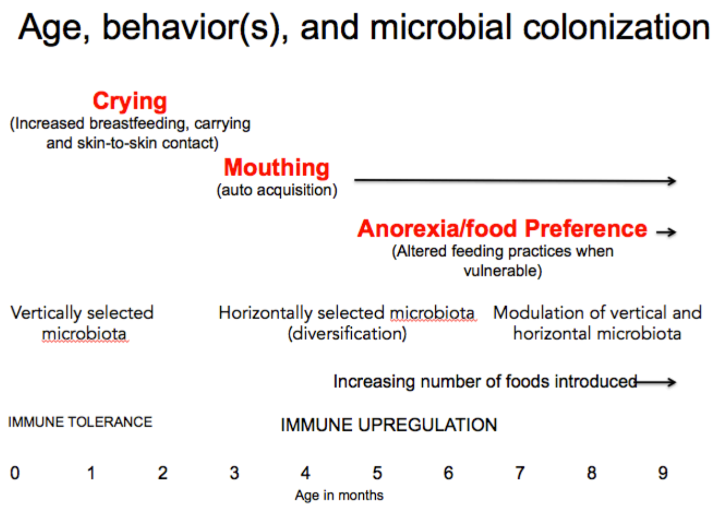


Figure 1: Age, infant behavior and microbial colonization.

This figure charts infant behaviors, differential microbial exposures and immune regulation over time. The infant crying curve increases in the first weeks of life, ensuring vertical transmission of bacteria from mother and other close relatives. Vertical microbial inheritance is key during this immunologically vulnerable window (ie naïve immunity). Mouthing and complementary feeding coincide with a necessary up-regulation of the immune system. Breastfeeding helps to stabilize and modulate horizontal exposures over time (Sheets and Worthman 2014)

> **H1:** The optimal ontogeny of the gut microbiome requires first a vertically-selected microbiota. As horizontal exposures increase, and with the continued protection of breastmilk over the first year(s) of life, the gut microbiome diversifies and stabilizes.
>
> **H2:** Contemporary human behaviors can interrupt the timeline of both vertical and horizontal exposures, resulting in altered microbial assembly, growth and health outcomes.

Both hypotheses highlight the importance of timing and context in development. The first underlines the successional nature of development, and requires a deeper exploration into the time-line of infant somatic growth and microbial development (Figure 1). The second hypothesis requires a broader understanding of how social, political, economic and ecological conditions may shape the micro-niches in which infants develop and interact. What are the social, behavioral or ecological factors that contribute to variations in microbial colonization, infant health, disease and growth patterns? These questions and hypotheses will be explored in Chapters 2-6.

The following section provides a historical and cultural context of the study population.

A political history of El Salvador and Joya de Cerén

The Republic of El Salvador is located in Central America, bordered on the north and east by Honduras, on the west by Guatemala, and on the south by the Pacific Ocean. As of 2013, El Salvador had a population of approximately 6.29 million, making it both the smallest and the most densely populated country in

Central America (Hernández and Munguía 2013). Over 85% of Salvadorans identify as "mestizo" or mixed Indigenous Native American and European Spanish origin, another 12% identify as mostly European descent, while the remaining 1% identify as purely Indigenous descent (*Pipil* or *Lenca*) (Central Intelligence Agency 2012). Very few Amerindians have retained their customs and traditions, having over time assimilated into the dominant Mestizo or Spanish culture. Low numbers of self-identified indigenous people may be partly explained by historically high rates of old-world diseases, absorption into the mestizo population, as well as mass murder during a 1932 Salvadoran peasant uprising (called "La Matanza," or massacre) where up to 30,000 peasants and indigenous people were killed and many others terrorized within a matter of days. Since La Matanza, indigenous populations in El Salvador have been reluctant to describe themselves as such, speak indigenous languages, wear traditional dress or be seen taking part in any cultural activities or customs that might be understood as indigenous ("History, Military Rule, 1931-1979" 2015).

Salvadoran history is replete with deep economic and social inequality, repression and corrupt government rule that led to centuries of violent revolts. After gaining independence from Spain in 1821, and from the Central American Federation in 1839, several Spanish Creole families formed an oligarchy that maintained control over the country's political and economic resources for nearly a century. A key figure in this effort was president Rafael Zaldivar (president from 1876-85), who aimed to insert El Salvador into the global economy through coffee exportation. Zaldivar enforced radical changes in land tenure that resulted in the expropriation of lands of indigenous communities and peasants, and the transferring of these lands to wealthy plantation owners (Valle 2015). These authoritarian regimes continued into the early 20th century when there was an international crash of coffee prices, and in 1932, a rebellion of peasants and workers exploded in El Salvador. The 1932 revolt, crushed by government forces in the “Matanza” massacre, ignited a new form of rule: a right-wing military dictatorship that employed political repression and limited reform to maintain power. This newly installed military government was also a political ally of the U.S. government in its mission to combat international communism. New land reforms, laws and institutions were inaugurated to maintain control over communities, to ensure economic productivity and to increase global market integration. This regime lasted until 1979, when a tumultuous period of political mobilization and violence (including the murders of Archbishop Romero and four American nuns in 1980) further fomented the organization of a leftist guerrilla movement known as the Farabundo Martí National Liberation Front (FMLN). The FMLN formed to fight the national government in what would be a prolonged and bloody civil war with over 75,000 fatalities. The US government supported the effort to prevent the left-wing guerillas from coming to power, providing over $7 billion in military aid and training (Rohter 1998).

In 1992 with the support of the United Nations, both parties signed the Peace Accords. These Accords have promoted the incorporation of the FMLN guerrilla group into the political system, the reform and reduction of the armed forces, and the implementation of a program of agrarian land reform. In 2009 the FMLN won the presidential elections with former journalist Mauricio Funes as its candidate. This same year the FMLN won the majority of the mayoralties in the country and a plurality of the National Assembly seats in municipal and legislative elections.

Unfortunately, the end of the civil war has not meant peace for this small, densely populated Central American country. During the 1980s roughly 25% of the Salvadoran population fled to the US and other countries seeking political asylum (Gammage 2007). In Los Angeles, a gang culture was established with the initial purpose of protecting the growing Salvadoran population from more established Mexican and African-American gangs (Elkus 2007). After the war, many high-ranking gang members were deported back to El Salvador, in a process that has been described as “unintentional state-sponsored gang migration” (Reynolds 2015). By 2005, El Salvador had 10,000 active gang members, and this number has only grown in the years since. Currently, there are 70,000 members of the MS-13 and 18th Street gangs operating in El Salvador, compared to approximately 24,000 Salvadoran police officers and 28,600 registered private security guards hired to protect Salvadoran individuals and firms that can afford them (RESDAL 2013).

Gang presence and violence permeates the country. In 2011, the government formed a truce with the gangs and violence decreased substantially. However, violent crime has steadily increased as the government supported truce dissolved between the two primary gangs. Refugees International suggests that roughly 90 percent of the country is under the de facto control of gangs, making El Salvador “one of the deadliest countries in the world” (Reynolds 2015). These gangs have increased their attacks both in urban and rural areas, a response to the government's rejection of their request for renewed dialogue. The current government has refused to negotiate with the gangs and insists on fighting the organizations with military force. Security operations against gang members often provoke retaliatory attacks on security personnel and citizens alike, the frequency and intensity of which fluctuate in response to the extent and severity of anti-gang operations. These Salvadoran gangs do not maintain a political ideology, but side with whatever party does not repress them, and homicide rates have continued to rise in El Salvador. A total of 1,399 homicides were reported in the first two months of 2016, an average of 23 homicides per day, making these the most violent months since the end of El Salvador's civil war (1980-1992). The national homicide rate has risen 118% percent in the first months of 2016, as compared to the same period in 2015 (Aleman 2016).

This situation influences the daily lives of Salvadorans in many ways. The frequency of public shootings and the gangs' access to military-grade weapons pose significant threats to the Salvadoran civilian population, although gangs employ a range of tactics and thus the influence is constantly changing. In addition to intra-gang violence and clashes with security forces, gangs in El Salvador are involved in extortion, drug trafficking, human trafficking, forced recruitment, abduction, carjacking, and other forms of violent and sexual crimes that are endemic throughout the country. For example, gang attacks against public transport workers, especially bus drivers, are common. Gangs heavily extort public transport workers, and the failure to pay extortion payments often results in violent and/or deadly attacks. These attacks are used to foment public outrage and force the government to enter into negotiation with gang leaders. The transportation stoppage can paralyze the capital and surrounding areas, and many businesses have closed or severely reduced working hours due to the risk of violence and reduction in secure transportation options for employees. Gangs often target a home or an apartment building simply because of their strategic locations, and once ordered out residents have no alternative but to leave (Reynolds 2015). This forced displacement happens with almost complete impunity and has led to increased immigration rates and displaced populations. Last year alone more than 32,000 unaccompanied Salvadoran children arrived at the U.S. border, many fleeing gang violence (Rogers 2015). The exodus continues now, though many more immigrants are stopped in Mexico as border-control efforts move south (Nazario 2015).

The effects of gang presence on the daily life of rural Salvadorans are erratic and always changing. Where guerilla tactics during the civil war aimed mostly at government targets with unintended civilian casualties, gangs utilize citizens for subsistence by extortion as well as for bargaining purposes. Changing dynamics within and between gang membership, as well as tactics with or against government forces affect how particular gangs interface with communities and individuals. Gang influence on my field site will be addressed in later sections after the local setting and study population have been described.

Community setting

Joya de Cerén is a small semi-rural community development nestled into the Zapotitán valley, located approximately 30 miles from the capital city of San Salvador. This region has a tropical climate with pronounced wet and dry seasons. Temperatures show little seasonal change, although the rainy season extends from May to October is referred to as “winter” time. Almost all of the annual rainfall occurs during this season. From November to April, the northeast trade winds control weather patterns creating a hot, dry and dusty climate. These hot winds circle the valley, trapping smoke from slash and burn agriculture as well as air pollution from nearby factories.

Joya was initially designed by the Institute of Rural Colonization (I.C.R) in the 1950s as an agricultural reform project. Additions to the community have been made since, in the way of dispersed settlements. According to the Salvadoran census of 2010, Joya has 6313 inhabitants. The population lives in 1049 households with an average of 6 people per household. Household and land density has increased dramatically as children mature and begin raising their own families. New households are built on family properties and there are commonly 2-5 households per property. Joya de Cerén has a dedicated public health worker and a public school that enrolls students from kindergarten through the 8th grade. Students who wish to continue on to study in high school must travel 4 miles to the closest town San Juan Opico.

Joya has one semi-paved street, although it is not the main thoroughfare. Calle “A” is a dirt and trash-packed road, lined with a grey-water sewage, and then multicolored fences or walls that surround each property, constructed of materials varying from tin to brick. During the day the streets have a constant flow of people going about their daily activities. Mobile street vendors call out their wares through megaphones and on loud speakers, and hungry dogs scour the street for scraps. Dusk falls at 6pm year round, and after dinner the streets are all but deserted unless there is a festival or holiday. The street is strewn with trash, despite government initiation of a twice-weekly trash pickup program. Many villagers feel the price of plastic trash bags is too high and are more accustomed to throwing trash on the street or burning it in their yard. During the dry season car and truck tires throw up a substantial amount of dust from the road, which mixes with smoke from trash fires, causing increased upper-respiratory conditions in children and adults.

From co-op to milpa to market: a socio-economic history of Joya de Cerén

To better understand the household social structure of Joya de Cerén, one must first understand the history of this planned community and the ongoing transformations in the economic system. Cultural anthropologist Carlos Lara conducted his field research on the social and economic history of this community in 1996-1997, and in the following section I draw from his research and my ethnographic data.

At the start of the 20th century, the lands that now make up the village of Joya de Cerén, formed part of a large hacienda owned by Dr. Francisco Dueñas.

In 1942 Dr. Dueñas sold over 3300 hectares of his hacienda to Junta Nacional de Defensa Social, asking that the land be used for agrarian reform. Slightly over a decade later, the Instituto de Colonización Rural (I.C.R.) founded the canton Joya de Cerén and built the first 80 houses on the divided properties, as part of an agrarian reform project (Lara 1998).

As part of this project, I.C.R. developed a cooperative association with the stated objective of bettering the life of the *campesino* through social and communal organization. The I.C.R. awarded agricultural lands to the community through the cooperative and the majority of workers in Joya de Cerén were members. Farmers received a salary for the work they did on the I.C.R. lands and received a dividend at the end of the harvest. At the start, lucrative sugar cane was the main crop grown on these lands. However, according to Lara (1998), farmers also established small plots of land on which to grow corn, beans, and vegetables (*milpas*) for subsistence to complement the low salaries from sugar cane work.

Seven years following its formation (in 1961), members moved to dissolve the cooperative and asked I.C.R. to divide the lands among the members of the community. The organization agreed and sold approximately 3.5-5 acres to each head of family at a price of approximately 3000 colones, or ($1200) per lot, payable over 20 years. Originally the titles prohibited the resale of lands, but later the law was modified, and so in this way Joya de Cerén was transformed into a community of small landowners.

Initially, these small-scale farmers continued producing cane sugar, but over time the production of corn and beans became more prevalent. As one informant suggested, “Before…almost everyone was producing cane, and few grew *milpa*, but today there is more *milpa* than cane, and others have sold their land” (Lara 1998). When Lara asked them why they had dissolved the cooperative, the old members and their children insisted that the workers only received a disproportionately small amount of the profit. Some townspeople blamed the I.C.R. authorities while others pointed to directors of the cooperative. But another important element affected the decision to dissolve the cooperative, which is the preference of the farmers to own private land. They wanted the agency to make decisions about what they needed and wanted to grow, and when they were given the choice, the great majority opted to grow basic grains instead of continuing to a more lucrative, mono-crop of sugar cane.

Thus, during this 40-year period, Joya de Cerén converted to a community of small-scale, mostly subsistence-based farms. When Lara conducted his field research in 1997, 84% of the head of families in Joya were using agriculture as a principal economic activity. At the time 64.2% of the families owned the land they lived on, and less than 30% of the farmers declared working on other people’s land. Upwards of 73% of the farmers of Joya were dedicated to the production of basic grains. Cane sugar represented only 11% and vegetables just under 6%. Subsistence farming formed the economic basis of this community. However, while agriculture was the primary source of subsistence in this population, it was not enough to sustain most families. Other economic activities in Joya complemented the small-scale farming, such as raising livestock and domestic animals. The majority of landowners owned between 4-6 head of livestock. And women often kept chickens, ducks, and pigs, both for food and for sale.

Lara documented the beginnings of an economic transition in Joya during the 1990s. He emphasized the shift of subsistence households from being fairly isolated economies that operated at the margin of the dominant monetary economy, to being increasingly well integrated into Salvador's capitalist market system. Although agriculture and subsistence farming were still the economic mainstay in Joya throughout the 1990s, pressures from global markets were beginning to induce changes on this local economy. Farmers who were selling part of their harvest were finding it increasingly difficult to depend on volatile market prices, as well as rapidly inflating costs of foods, goods and education. Subsistence farming in some ways helped to offset this economic instability. However, as subsequent generations grew, agricultural lands were being sold or divided up amongst large families into subplots or "*mini fincas*" that no longer could satisfy the total necessities of families.

The fragmentation of the agricultural property had negative repercussions on the agricultural production and livestock of the community, and with each generation the smaller plots of land meant less subsistence provision for larger families. This resulted in younger generations searching out new sources of income, particularly in the formal sector of the market economy to complement the decreasing output from the *milpa* and to survive in the national economy. By 1997, 88% of young people no longer saw paid work as a supplemental activity, but rather they viewed it as full time work. This is not to say that 88% of young men were employed in the paid work force; in fact at the time, 80% of the 10-29 year olds were still active in the agriculture and livestock, and only 19.8% were working in the industry and service sector. However, Lara revealed a generational and gender-specific transition from subsistence farming into a wage-labor market.

Economic transition and gendered labor markets

An important economic activity in Joya that emerged in the 1990s was the ownership of small food stores throughout the community. Because there was no commercial zoning in the original plans for Joya de Cerén, these stores are family-owned and located within homes. In most town centers in El Salvador, commercial zoning centers around the town square, as well as throughout residential districts. Instead, the only dedicated store in Joya de Cerén is an agro-servicio, which supplies the town with hardware and agricultural products. Joya is physically far from commercial districts (8.5 mi) or town centers (4 mi) such that these small home-stores emerged to fill the need for basic necessities including soda, snack foods, soap, sugar, salt, sweet breads, diapers and dried soups. Chicken, eggs and dairy products are sold at only a few stores, and produce is sold in fewer still.

Residents purchase produce from two ambulatory venders who sell vegetables out of the bed of their trucks, shouting through loud speakers as they drive slowly up and down the dirt roads. Other small food enterprises work out of their houses during mealtimes. During lunch and dinner, some families who live on the main roads set up a table on which they sell homemade tortillas, *pupusas* (a national dish made with a tortilla of corn or rice filled with meats, beans and cheese), French fries or fried yuca available seasonally. Women often supervise and run these informal businesses as an extension of their domestic labor and chores, or supervise older children who attend customers. It has become such a part of these family's lives that often it is not recognized as paid work.

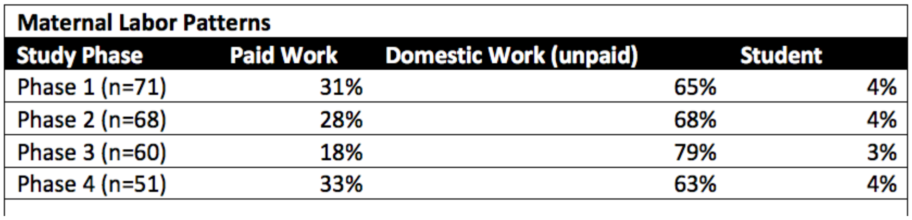
Income received from family members living abroad constitutes another important economic contribution to many households in Joya. In the 1990's, 37% of the families received such economic support (Lara 1998). In my study, 18% of families admitted to having external support from family in the US. The majority of the families who received remittances were sent between 100-200 dollars US per month, which represented a substantial increase to household income. Lara reported that 50% of these households considered this income to be fundamentally important for household basics, 25% was used for agricultural expenses, and 12.5% for children's education. The final 12.5% used the money to pay debts and other odds and ends, although it was difficult to determine this category of expense (Lara 1998).

A major change in the political economic landscape of this region emerged slightly after Lara conducted his investigation on Joya residents. Following the end of the civil war in the mid 1990s, a boom of foreign-owned factories producing textiles and paper-goods called "maquiladoras," began developing the

Zapotitán valley near Joya de Cerén. Recent estimates indicate the immensity of the industry, which currently employs over 74,000 people. This industry nationally represents 12 percent of the 636,000 jobs in the private sector (Ayala 2015). This industry almost exclusively hires women, and makes up 80 percent of the total formal market sector for women in the country (FIDH 2005). Factories prefer to hire women over men, because they are believed to work harder, show a higher level of patience and dexterity, and work for considerably less money than men in performing repetitive factory work (Jorgensen 2017; Domínguez et al. 2010).

The overall effect of these factories on gender inequality is hotly contested. Some scholars have argued that the factory sector has offered women alternatives and an opportunity to work in the formal sector that was previously unattainable (Lim 1990). Others suggest that factory work can build women's self-esteem and increase the ability to make independent choices (Kabeer 2004). In contrast, Domínguez et al. (2010) argue that gender relations have changed little since the proliferation of the factories. These scholars suggest that the extraordinarily low-income levels earned by female workers supports only survival, but do not allow them to challenge patriarchal gender hierarchies. Current study data from my interviews with subjects and informants alike suggest that all of these perspectives may be true, depending on SES. Nonetheless, transformations in labor practices have had enormous influence the lives of men, women and children in Joya de Cerén. Most relevant to this dissertation is the ways in which these gendered labor practices influence daily schedules and childcare practices in Joya de Cerén. This will be addressed in Chapter 3-5 with an overall synthesis in the conclusion.

Economic activity and the division of labor in each household have been and continue to be based on variables such as gender and age. The responsibility of economic provision for the family was traditionally a male role in this region. Men provided the material necessities to all members of the domestic group, mostly through subsistence and paid work in agriculture. Agriculture was and continues to be considered a man's domain: fathers would teach their sons about subsistence agriculture. Now that this population is largely integrated into the market economy, only older men continue to work small *milpas*.

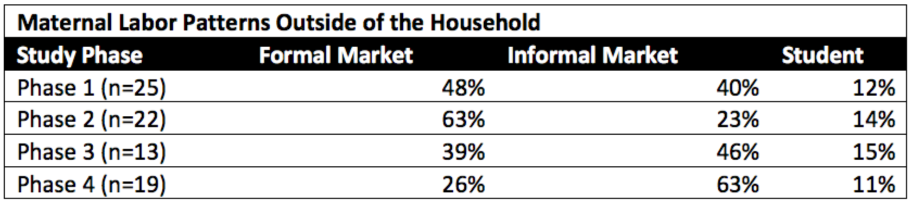


Maternal Labor Patterns

Study Phase	Paid Work	Domestic Work (unpaid)	Student
Phase 1 (n=71)	31%	65%	4%
Phase 2 (n=68)	28%	68%	4%
Phase 3 (n=60)	18%	79%	3%
Phase 4 (n=51)	33%	63%	4%

Table 1: Maternal Labor Patterns.

This table shows the percentages of mothers who work outside of the home for pay, those who stay at home full-time, and students. If a mother works outside of the home it does not mean that she is not mainly or solely responsible for unpaid domestic work at home.



Maternal Labor Patterns Outside of the Household

Study Phase	Formal Market	Informal Market	Student
Phase 1 (n=25)	48%	40%	12%
Phase 2 (n=22)	63%	23%	14%
Phase 3 (n=13)	39%	46%	15%
Phase 4 (n=19)	26%	63%	11%

Table 2: Maternal labor patterns of mothers who work outside of the house.

This table shows the breakdown of formal and informal market participation amongst mothers who participated in paid work or are students. Formal market jobs most often consist of factory work and bank telling. Informal market jobs often constituted selling goods, foods or services. Higher levels of formal employment in the first months of an infant's life were likely a result of factory jobs that offered women 3 months' maternity leave (usually partial pay). After these three months' mothers often left their jobs and turned to more informal market jobs before returning to work once their child was older.

Women were traditionally expected to stay home, maintain the house, and raise the children. Chores included washing clothes, cooking, tending to domestic animals, milling corn and taking caring of young children, among other activities that women perform each day. Lara reported that in 1996-97, 77.9% of the women interviewed said they did not work for pay, but were dedicated to domestic tasks. Although results from the current study did not show dramatic increases in women's participation in paid work, this sample does not likely reflect the general labor trends of Joya. This study showed that anywhere between 63%-79% of mothers did not work for pay outside of their homes (see Table 1). However, this sample contained only women with infants and young children, and it is likely that the proportion working for pay among the general population of women is higher than these figures imply.

At the same time, sample demographics (in Table 1) indicate interesting patterns in labor participation amongst women of this sample. Formal market sector work for women of this age range most commonly comprises factory jobs, work in the commercial sector, or paid domestic service. More commonly, work is in the informal market sector (selling foods and goods from homes or a roadside stand set up for certain hours of the day), or service sector (cleaning laundry or other services for pay). Table 2 illustrates the instability of women who work in both the informal and formal markets over the study period. Although there is a small portion of workers who find semi-permanent positions, much of the work is contract-based and many individuals remain underemployed. Study participants with the most secure jobs often cite poor working conditions and ill treatment from supervisors. Workers suggest zero flexibility for illness or other life events, no benefits, exhausting hours and unhealthy working conditions. Integration into the market economy has made for an unstable economic and destabilizing social foundation for this community.

Factory work is highly competitive and sought after for young women. This is not because it is easy work, secure, or good pay, but because it is considered the best of all options in the region. While mistreatment and poor working conditions are experienced daily, it is often the steadiest work available for single and educated women. Importantly, however, factories only hire women between the ages of 18-30. This means that only women of childbearing years are considered employable in these factories, which may help contribute to job instability and insecurity among formal sector mothers in this study (see Table 3 and 4). According to study participants, pregnant women are systematically fired because the *maquilas* do not want to offer benefits and pay the obligatory three-month maternity leave. Additionally, inflexible and long working hours, low pay and poor treatment contribute to difficult working conditions for mothers of young children. Finally, mothers often pointed out that the cost of childcare combined with the price of infant formula were higher than a monthly salary and make staying home a more feasible option in many circumstances. Mothers with larger child care networks tended to continue working at their factory jobs after the 3-month maternity leave.

According to mothers, all of the factories are not equal. Increasingly, some factories provide better working conditions and benefits. For example, one mother in this study worked in a factory that had a program for young mothers to pump and store breast milk. The program offered mothers two breaks during the day for pumping and mothers were able to store the milk in dedicated refrigerators. This allowed the participating mother to spend less of her monthly salary on infant formula. Unfortunately, this program is unique in this area, and most women agree that working conditions while caring for young infants is impossible without good family support. Taken together, these insights help explain both the low rates of formal employment and job instability among working mothers of Joya.

Many mothers preferred the informal market sector to the formal for the main benefit of flexible working hours. Mothers filled in where needed such as washing a neighbor's clothes, cooking something to sell, or managing a small store. In many cases these mothers continued caretaking for their young children while they worked or supervised older children as they took care of the infant.

Men of all ages in this region experience severe underemployment, although my study population may not reflect its extent. Fully 62% of fathers in this study are employed full-time, 18% are employed part time, 3% were unemployed and looking for work, 1% was disabled and out of work, and the employment status of 16% was unknown because they were uninvolved with the child. These statistics are not likely representative of the broader population of men in Joya de Cerén because I was guided away from working directly with gang-associated families. Almost all gang members are unemployed in the formal sense, but live off extorted money from their neighbors and other gang activities.

The social realm

Transformations in the economic life of Joya residents have affected the social world as well. Where subsistence economies value relationships and social life as part of the socio-economic system, the market economy demands the subordination of social life to economic interests. Conversely, subsistence farming encourages human resources to go into the development of social relations over and above economic benefits. Joya has seen this shift as the majority of the population has now integrated into the market economy. Market integration has disrupted traditional roles and power dynamics by affording women and young people a new earning power that older generations and many men do not have. Whereas the domestic group has been the primary unit wherein cooperation and mutual support are realized amongst townspeople of Joya de Cerén, the structure of the domestic unit is changing dramatically towards a nuclear or single-parent system.

Integration into the market economy also has led to an increased stratification of socioeconomic status (SES) among households in Joya de Cerén. Ultimately, a small group of individuals has risen in socioeconomic level above the remainder of the community. For example, there are some teachers who work for the ministry of education or health and earn a salary that is far superior to that of the rest of the community. These individuals also gain prestige in the community. There are other community members who make superior incomes such as small business owners, owners of the corn mill, the owner of the *conjunto musical Joya de Cerén* and the owner of a passenger bus, all of whom make more money than other members of the community. Additionally, there are individuals or families who receive large remittances from family members abroad. One elderly lady who lives in Joya has two sons who rank high in the Salvadoran military. Her house is beautifully constructed, with well-manicured grounds and 2-5 armed soldiers that stand guard outside her home at all hours. In general, these wealthier community members live in better-constructed dwellings with more services and amenities than others living in town. These SES differences have more greatly stratified the community than in previous years.

The transition from agriculture to market economy also has led to a demographic transition where women have far fewer children than in prior generations. Fertility has dropped 60% in the last 30 years, from 6.3 lifetime births per woman in the mid-1970s to 2.5 by 2008 (Ministerio de Salud El Salvador 2009). Although rural women in El Salvador on average have 3 children compared to urban women who have on average 2.1 children (Ibid), women of this study had an average of only 2 children. This demographic transition affects family dynamics, child and elderly care networks, and other family dynamics discussed in chapter three.

Finally, the rampant gang culture is not entirely a product of this economic transition, however impoverished circumstances certainly fuel gang memberships among young men. The effect of gangs on the daily life of Salvadorans has been previously discussed, and cannot be underestimated. These activities change the ways in which residents of Joya de Cerén conduct business, how they utilize public spaces, with whom they share information, how families interact, and who they trust.

Although there have been many other social manifestations from the transition from subsistence agriculture to market integration, the above section details those most closely related to the topic of this dissertation, which is the microbial ecology and development of the microbiome. Chapter three of this dissertation will more clearly outline these changes and how they are related to the care of infants and relationships to microbial development.

Selection of study site

Joya de Cerén was chosen as a field site for multiple reasons. The complexity of conducting biocultural, microbiomics research required a deep research infrastructure. Long-term, familial relationships with villagers, community leaders, university contacts (in San Salvador), and public health officials made the acquisition of permission and research collaboration tenable. The promise of collaboration with the health promoter, Niña Ana Carmen Diaz and her team shortened the start-up and recruitment time substantially, as well as vastly improving the quality and accuracy of research. Collaboration with Niña Ana lent immediate credibility and opened the doors of homes in the community. The trust and respect she had earned within the community supported my longitudinal efforts and allowed me to build trusting relationships with study participants that would otherwise be difficult.

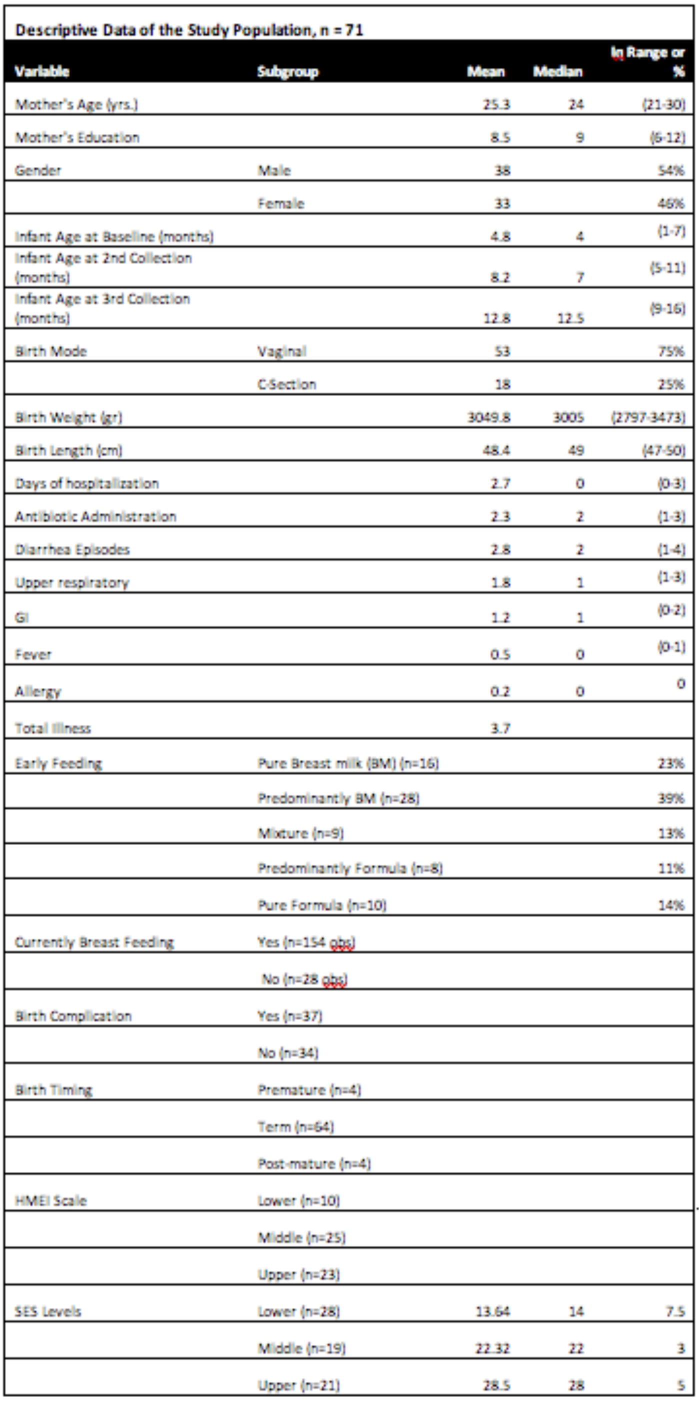
Living in my father's research house allowed me to secure a deep freezer for the proper -20 degree below zero storage of the microbial samples. Personally paying the electricity bill, I could better ensure that the electricity would not be cut off, which could render hard-earned and time-sensitive microbial samples useless. I also was able to increase security measures on the house to ensure my safety, a lower likelihood of theft or tampering with my research data and equipment. The proximity to San Salvador, and the Comalapa International Airport was also an attractive factor, as it ensured the rapid and safe transport of microbial samples. Finally, and in many ways most importantly, this impoverished, semi-rural, market-integrated community represents many others in developing countries around the world. Surprisingly, these demographics have been largely been ignored by microbiomic researchers.

Having close family contacts and support also ensured my safety in a socio-politically unstable country. In March of 2012 a truce was made between the two rival gangs in El Salvador. The accompanied decrease in violence was an encouraging sign during my preliminary investigation in September 2012. Unfortunately, by the time I began my research in September 2013, the truce had fallen through and violence rates surged. With the support of family and friends I decided to continue my study, as they helped me keep abreast of local and national news that would affect my personal safety. Now knowing how quickly the situation has deteriorated, I realize how fortuitous and favorable my window of research was. My family and personal history in Joya de Cerén afforded me a particular access to conduct my research in this community and in El Salvador in general.

Study population and methods

Participants were originally enrolled through Niña Ana Carmen Diaz, the health promoter, beginning in September 2013. At their monthly visit to the health promoter's office or upon Niña Ana's home visit, mothers were told about the study and invited to participate. In the first study phase, 60 mothers and infant pairs were enrolled, although one dropped out before the study began saying she was not interested. At the beginning of each of the three subsequent study phases, additional mother-infant pairs were enrolled. In all, 71 primary caregivers and their infants completed at least one phase of investigation. Eight mothers dropped out of the study for different reasons: 2 mothers decided to discontinue for unspecified reasons; one mother was dropped from the study because of her husband's heavy gang involvement; one infant passed away; and 4 families moved away from the area. All infants were between the ages of 0 and 12 months at baseline (average age at baseline was 4.8 mo) (see Table 3).

The original intent was to recruit 60 infants between 0-4 months at baseline. Unfortunately, there were not enough infants of this age range in this community to accommodate this target sample size, particularly accounting for attrition rates. Thus, the age of enrollment was broadened, and with every subsequent sample collection phase new infants were recruited to maintain high sample size. Although these baseline age ranges complicated longitudinal analyses, it also provided a holistic view of one community, as virtually all infants of this age range in this community were represented in the study.



Descriptive Data of the Study Population, n = 71

Variable	Subgroup	Mean	Median	In Range or %
Mother's Age (yrs.)		25.3	24	(21-30)
Mother's Education		8.5	9	(6-12)
Gender	Male	38		54%
	Female	33		46%
Infant Age at Baseline (months)		4.8	4	(1-7)
Infant Age at 2nd Collection (months)		8.2	7	(5-11)
Infant Age at 3rd Collection (months)		12.8	12.5	(9-16)
Birth Mode	Vaginal	53		75%
	C-Section	18		25%
Birth Weight (gr)		3049.8	3005	(2797-3473)
Birth Length (cm)		48.4	49	(47-50)
Days of hospitalization		2.7	0	(0-3)
Antibiotic Administration		2.3	2	(1-3)
Diarrhea Episodes		2.8	2	(1-4)
Upper respiratory		1.8	1	(1-3)
GI		1.2	1	(0-2)
Fever		0.5	0	(0-1)
Allergy		0.2	0	0
Total Illness		3.7		
Early Feeding	Pure Breast milk (BM) (n=16)			23%
	Predominantly BM (n=28)			39%
	Mixture (n=9)			13%
	Predominantly Formula (n=8)			11%
	Pure Formula (n=10)			14%
Currently Breast Feeding	Yes (n=154 obs)			
	No (n=28 obs)			
Birth Complication	Yes (n=37)			
	No (n=34)			
Birth Timing	Premature (n=4)			
	Term (n=64)			
	Post-mature (n=4)			
HMEI Scale	Lower (n=10)			
	Middle (n=25)			
	Upper (n=23)			
SES Levels	Lower (n=28)	13.64	14	7.5
	Middle (n=19)	22.32	22	3
	Upper (n=21)	28.5	28	5

Table 3: Descriptive statistics on study population.

Early feeding was measured in infants 6 months and under. Early hospitalization was largely days after birth that infants were kept at the hospital for observation, although in one case the infant was released but was then readmitted for a week beginning at day 4.

Mothers were between 15-42 years old, with an average age of 25. Mothers had on average attended 8-9 years of school. Approximately one-quarter of the infants were born via C-section. Males made up 54% of the infant population (see Table 3).

The study was conducted in five phases including baseline, during which caretakers were visited at home and formally interviewed, however only three phases are represented in this study. At least one fecal sample was collected per infant during the course of each study phase, with additional sampling around key events (e.g. administration of antibiotics, severe illness, etc.). After the interview, caregivers were asked about the health history of their child since the last visit, and a 24-hour dietary recall. Return visits to households were made to complete at least two to three 24-hour recalls per infant, per study phase. Anthropometric measurements of infant weight and height were collected in each study phase, as well as birth statistics at baseline. Certain infants who were below weight or commonly sick were more closely monitored with more frequent sample collections and measures taken. Extensive participant observation during meal times and other caregiving activities was conducted in a subgroup of households. This subgroup of households was also sampled environmentally, which consisted of swabbing common areas where infants commonly touched, as per the protocol of the Sloan project and the Dominguez-Bello laboratory.

Research assistants delivered disposable diapers and zip lock bags to caretakers the night before a sample was collected. Soiled diapers were collected within two hours of infant defecation; otherwise they were disposed of and mothers given a clean diaper. Samples were collected with sterile swabs and stored in a deep freezer at -20 Celsius for under three months before being hand carried still frozen to the Knight Laboratory at the University of Colorado at Boulder for sequencing.

Bacterial DNA was extracted from the samples using the Eppendorf EpMotion 5075 robot. Polymerase chain reaction (PCR) was then used to amplify the 16S ribosomal RNA (rRNA) genes to characterize the taxa present in the gut microbial communities of these infants (n = 215 samples successfully analyzed). The 16S rRNA gene (often referred to as "16S") is widely used in studies of microbial ecology as a "barcode gene" (Hebert et al. 2003) to quantify microbial community structure and diversity (Pace 1997; Hugenholtz et al. 1998). The widespread use of 16S as a barcode gene has been driven in part by the fact that it is universal across bacteria and archaea, and can be easily amplified from a wide diversity of taxa at one time by the polymerase chain reaction (PCR). For these reasons the 16S gene is phylogenetically informative, because it can be used to identify and phylotype sequences based on extensive databases of 16S sequences with associated taxonomic and phylogenetic information (Pace 1997; Woese and Fox 1977). There are numerous advantages to using 16S as a microbial community barcode gene, but also numerous disadvantages including amplification and sequencing bias and error (Suzuki and Giovannoni 1996; Hong et al. 2009), and difficulty with the accurate taxonomic identification and binning of short sequences (Qin et al. 2010; Kunin et al. 2010; Haas et al. 2011; Rodrigue et al. 2010).

Samples were then sequenced on the Illumina second-generation sequencer. Alpha diversity was calculated using Chao1 diversity index based on the rarefied OTU table at a depth of 1226 sequences per sample. Alpha diversity is a summary measure of intra-sample taxonomic diversity, which captures both the organismal richness of the sample and the evenness of the organisms' abundance distribution. The calculations were performed either with the QIIME pipeline (Caporaso et al., 2010), with R software (R Foundation for Statistical Computing, Vienna, Austria) using the vegan (Oksanen et al., 2011) and picante (Kembel et al., 2010) packages, and/or JMP Pro 12 software. To compare between-sample variations in the composition of the total microbial community, unweighted and weighted UniFrac distances (Lozupone and Knight, 2005) were calculated with the QIIME pipeline (Caporaso et al., 2010). PCoA (Principal Coordinate Analysis) was performed on both distance matrices and coordinates were used to draw 3D graphical outputs.

Roadmap of dissertation

This dissertation is organized into 6 total chapters. Chapter 2, "*The Early Development of the Infant Microbiome in a Semi-rural Salvadoran Population,*" explores the vertical selection of maternal-infant interactions and microbial selection. This chapter explores how the interruption of this vertical selection through birth mode and early feeding practices introduces microbial variation. This chapter is a more traditional representation of current microbiomics research, analyzing well-established variables that influence early development, for the ultimate purpose of comparing cross-culturally for future publication. It also serves as a focused exploration of maternal-infant selection factors, compared to the following chapters. Chapter 3, "*A Developmental Ecology of The*

Infant Gut Microbiome," begins to explore the horizontal basis of microbial selection. This chapter broadens the scope of current investigation to incorporate the social, political economic and environmental variables that influence early micro niche production and microbiota development. This chapter charts new territory in microbiomics research, and in doing so, develops a multi-level model from which to imagine the influence of broader processes on early exposures and development in this population and others. Chapter 4, "*Infant Growth, Health and the Gut Microbiome,*" examines how both vertical and horizontal factors influence gut microbiota and growth/health outcomes. This chapter argues that the developing microbiome evolved in part to support the high energetic demands for growth of the human infant during the early months of life, and that a disruption of this development should be reflected in divergent patterns of infant growth. Chapter 5, "*Functional Index of Relevant Taxa,*" presents current knowledge about the form and function of the most common microbes in the study population. Beneath the description of each microbe is a deeper investigation of the current study results. This section explores the factors that affect exposure and developmental patterns, as well as defining predictive ranges of microbial development over time. Investigating outlier infants provides a profound understanding of the tri-fold ecological-microbial-host interactions over time. The conclusion (Chapter 6) of this dissertation provides a synthesis of this multidisciplinary research. This chapter briefly summarizes study findings, draws connections among the different levels and chapters, revisits hypotheses, and summarizes the major limitations of this study. Finally, policy and future research recommendations are made. Ultimately, the findings presented in this dissertation support the need to utilize an integrated, multi-level model that is grounded in anthropological knowledge in order to better understand the complex process of human-associated microbial ontogeny.

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CHAPTER 2: THE EARLY DEVELOPMENT OF THE INFANT GUT MICROBIOME IN A SEMI-RURAL SALVADORAN POPULATION

Introduction

Within the human intestinal tract lives a dynamic community of over 100 trillion microbes called the intestinal microbiome (Turnbaugh et al. 2007; Xu et al. 2007). Ideally, these microbial inhabitants initiate colonization at birth (Palmer et al. 2007), and successionally flourish into a complex microbial ecosystem within the first years of life (Dominguez-Bello et al. 2011). It has become increasingly clear however, that interruptions during this early developmental period can have detrimental consequences for human pathophysiology and disease susceptibilities throughout the life course. Human-associated microbiota play central roles in nutrition and metabolism, immune development and regulation, and protection from pathogens (Hooper et al. 2001; Maslowski and Mackay 2011). The list of diseases associated with a dysbiotic, or imbalanced, microbiome continues to grow, and has been linked to later infectious diseases (Russell et al. 2010; Honda and Littman 2012), metabolic disorders (Turnbaugh et al. 2006; Ley 2010), and autoimmune and inflammatory conditions (Honda and Littman 2012; Sellitto et al. 2012), among others.

The early development of the gut microbiome is thought to be a successional process. In other words, *which* microbes colonize the infant gut and *when*, may have long-term health consequences (Palmer et al. 2007; Vael and Desager 2009). Although many of the initial microbes are transient, these signature species alter the intestinal environment in ways that lead to more complex and stable ecological systems. From an evolutionary perspective, the birthing process, followed by exclusive breast-feeding, represent the infant's initial seeding of a "vertically" inherited microbiota. Shortly after birth, facultative anaerobes including Enterobacteriaceae, *Streptococci* and *Staphylococci*, colonize the infant body. These bacteria establish an anaerobic luminal environment in the intestine, preparing the gut for the subsequent appearance of important obligate anaerobes such as *Bifidobacterium* (Adlerberth and Wold 2009). Blanton et al. (2016), recently discovered that mice recipients of microbiota from 6-month-old infants grew more than recipients of microbiota from 18-month-old infants, despite identical diets. This research illuminates the existence of developmentally distinct signature microbiota, optimized at each stage of development to satisfy the needs of a growing infant.

The interruption of these key events can cause abrupt shifts in abundance or representation of specific taxa, altering intestinal development, and the succession thereafter (Blanton et al. 2016; Dominguez-Bello et al. 2010). Experimental rodent studies have shown that early exposures and interruptions can have short and long-term effects on the immune system and metabolism. One study reported that even after microbiota return to "normal" population structure (after an antibiotic-induced disruption), elevated adiposity emerged in early adulthood (Cox et al. 2014). These lasting metabolic consequences provide evidence for an early life "critical window" of host-microbe metabolic interaction, during which metabolic profiles are developed for the remainder of the life course (Livanos et al. 2016; Cox et al. 2014).

Neonatal care and medical practices have become central to the investigation of this phenomenon, due to associated changes in infant microbial assembly. Birth mode (i.e., cesarean section or vaginal) (Dominguez-Bello et al. 2010), early feeding practices (breast and/or formula feeding) (Blaut and Clavel 2007), hospitalization (el-Mohandes et al. 1993; Schwiertz et al. 2003) and antibiotic administration (Jernberg et al. 2007), are associated with the interruption of this early microbial development in studied populations.

Researchers are beginning to explore connections between epidemic rates of chronic disorders in developing countries and changes in the microbiota of these populations. Yet little is known about the intestinal microbiome of populations outside of high-income, developed countries (Kabeerdoss et al. 2013). Current understandings of microbiome development in neonates from developing countries comes from limited, culture-based studies conducted over four decades ago (Mata and Urrutia 1971; Albert et al. 1978). Neonates from low-income countries experience different early exposures than those of wealthy countries. Sanitation and water systems, different infant care practices and medical systems can all contribute to variation in exposure during early life. The present study was undertaken to examine the development of the gut microbiota in a semi-rural Salvadoran infant population. Using molecular techniques directed at the 16S rRNA gene, this chapter first elucidates the foundational characteristics of microbial assemblage, and then explores and tests the expected factors of microbial development to determine significance.

To explore these factors, this chapter utilizes a developmental ecology framework. This perspective offers an integrative orientation for understanding the effects of early life exposures on developmental processes (Worthman 2010). The framework enables researchers to draw concrete connections between developmental processes and proximal conditions by operationalizing comparative research within populations (Ibid). Moreover, this framework emphasizes theoretical specificity and facilitates the generation of testable hypotheses (Worthman 2010; Whiting et al. 1975). This chapter draws from this rich and established body of research to explore the intra-population variation of microbial colonization based on age, birth mode, and early feeding in a Salvadoran infant population.

Study population and context

Joya de Cerén is a small semi-rural community located approximately 30 miles from the capital city of San Salvador, El Salvador. Located in a lowland valley, this region has a tropical climate with pronounced wet and dry seasons. Over 85% of the population identify as “mestizo” or mixed Indigenous Native American and European Spanish origin, another 12% identify as mostly European descent, while the remaining 1% identify as purely Indigenous descent (*Pipil* or *Lenca*). The community was initially designed in the 1950s as an agricultural reform project and additions to the development have been made since, in the way of dispersed settlements, currently totaling 6313 inhabitants. This population has largely transitioned from subsistence-based agriculture to a struggling market economy. Households are poor, with an average of 6 people living in each dwelling. Unemployment rates are high, and on average only 1-2 adults financially support the entire household.

The environmental, socio-ecological and political economic conditions of the study population resemble other densely populated, developing countries, and thus this community provides an attractive model to begin assessing the affect of environmental variables on the infant gut microbiota.

Research Questions and Hypotheses

The main research questions of this chapter are as follows: *what is the overall microbiome composition of this infant population*; and *how do early care practices affect the composition and development of the intestinal microbiome in this Salvadoran infant population?* This section outlines the hypotheses based on findings from extant literature that will be tested in this chapter.

H0: Overall composition

H0a: A-diversity should increase steadily with age, a shift that will be associated by distinct bacterial assemblages at each age group.

H1: Birth mode

H1a: Alpha diversity should be higher in cesarean delivered compared to vaginally delivered infants at all ages.
H1b: Vaginally delivered infants should reflect a vertically inherited microbiota including higher levels of *Bifidobacterium, Lactobacillus*, and

Streptococcus. Cesarean delivered infants will have a more horizontal exposure reflected in their microbiota, including skin and environmental microbes including *Corynebacterium*, *Dermacoccus*, and *Enhydrobacter*.

H2: Early feeding

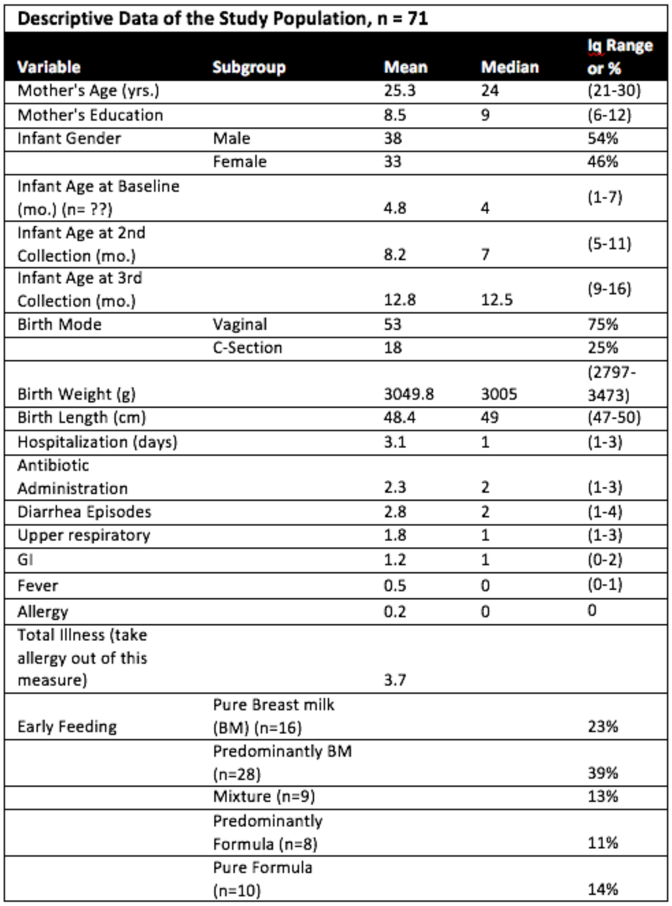
H2a: Alpha diversity should be higher in formula fed infants compared to breastfed infants.
H2b: Breastfed infants should reflect breast-milk-associated microbiota, including higher levels of *Bifidobacterium, Lactobacillus, Megasphaera* and *Veillonella*. Formula fed infants should harbor bacteria associated with cow's milk-based formula including *Blautia,* Lachnospiraceae, and Enterobacteriaceae.

H3: Birth-mode and early feeding interact as synergistic factors, either contributing to enhanced levels of anti-inflammatory, age-appropriate and breast-milk associated microbes, or together instigating dysbiosis, instability, delayed colonization and increased levels of pro-inflammatory microbiota.

H3a: A-diversity should be lowest in the VDBF infants at 0-3 mo., increasing steadily over time, becoming the most diverse sub-group by 12+ mo.
H3b: Vaginally Delivered, Breastfed (VDBF) infants should harbor a vertically-inherited microbiota, replete with anti-inflammatory, age-appropriate and breast-milk degrading bacteria, including higher levels of *Bifidobacterium, Lactobacillus, Megasphaera* and *Veillonella*.
H3c: Cesarean Delivered, Formula fed (CDFF) infants should have dysbiotic, and unstable microbiomes that show delayed colonization patterns with increased levels of pro-inflammatory microbiota including *Blautia,* Lachnospiraceae, and Enterobacteriaceae.

Methods

To explore the outlined hypotheses and demonstrate gut microbiome signatures characteristic to each age group during the first two years of life, this study sequenced infant fecal samples using the 16S gene. Study participants included 71 infants from Joya de Cerén, between the ages of 0 and 12 months at baseline (average age at baseline = 4.8 mo) (see Table 4). Interviews were conducted with mothers and/or primary caretakers, where at least three fecal samples were collected per infant over the course of a year, with additional sampling around key events (e.g. administration of antibiotics, severe illness, etc.). Disposable diapers were delivered to caretakers the night before a sample was collected, and soiled diapers were collected within two hours of infant defecation. Samples were collected with sterile swabs and stored at -20° Celsius for up to three months before being packaged on ice and hand-delivered to the Knight Laboratory at the University of Colorado at Boulder for sequencing.



Descriptive Data of the Study Population, n = 71

Variable	Subgroup	Mean	Median	Iq Range or %
Mother's Age (yrs.)		25.3	24	(21-30)
Mother's Education		8.5	9	(6-12)
Infant Gender	Male	38		54%
	Female	33		46%
Infant Age at Baseline (mo.) (n= ??)		4.8	4	(1-7)
Infant Age at 2nd Collection (mo.)		8.2	7	(5-11)
Infant Age at 3rd Collection (mo.)		12.8	12.5	(9-16)
Birth Mode	Vaginal	53		75%
	C-Section	18		25%
Birth Weight (g)		3049.8	3005	(2797-3473)
Birth Length (cm)		48.4	49	(47-50)
Hospitalization (days)		3.1	1	(1-3)
Antibiotic Administration		2.3	2	(1-3)
Diarrhea Episodes		2.8	2	(1-4)
Upper respiratory		1.8	1	(1-3)
GI		1.2	1	(0-2)
Fever		0.5	0	(0-1)
Allergy		0.2	0	0
Total Illness (take allergy out of this measure)		3.7		
Early Feeding	Pure Breast milk (BM) (n=16)			23%
	Predominantly BM (n=28)			39%
	Mixture (n=9)			13%
	Predominantly Formula (n=8)			11%
	Pure Formula (n=10)			14%

Table 4: Descriptive statistics of study population

Bacterial DNA was extracted from the samples using the Eppendorf EpMotion 5075 robot. Polymerase chain reaction (PCR) was then used to amplify the 16S ribosomal RNA (rRNA)[1] genes to characterize the taxa present in the gut microbial communities of these infants (n = 215 samples successfully analyzed). Samples were then sequenced on the Illumina second generation sequencer. Because bacterial loads could not be effectively controlled for because of differences in the sampled area or volume, shifts in bacterial community structure and alpha diversity are the key parameters explored in this study.

Alpha diversity[2] (α-diversity) was calculated using Chao1 diversity index based on the rarefied OTU table at a depth of 1226 sequences per sample. The calculations were performed either with the QIIME pipeline (Caporaso et al., 2010), with R software (R Foundation for Statistical Computing, Vienna, Austria)

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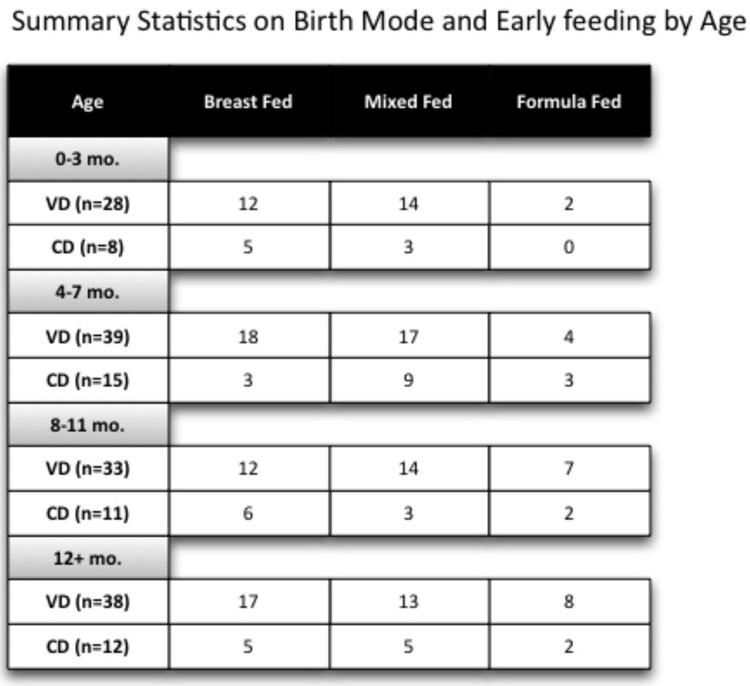
[1] The 16S rRNA gene (often referred to as "16S") is widely used in studies of microbial ecology as a "barcode gene" (Hebert et al. 2003) to quantify microbial community structure and diversity (Pace 1997; Hugenholtz et al. 1998). The widespread use of 16S as a barcode gene has been driven in part by the fact that it is universal across bacteria and archaea, and it can be easily amplified from a wide diversity of taxa at one time by the polymerase chain reaction (PCR). For these reasons the 16S gene is phylogenetically informative, because it can be used to identify and phylotype sequences based on extensive databases of 16S sequences with associated taxonomic and phylogenetic information (Pace 1997; Woese and Fox 1977). There are numerous advantages to using 16S as a microbial community barcode gene, but also numerous disadvantages including amplification and sequencing bias and error (Suzuki and Giovannoni 1996; Hong et al. 2009), difficulty with the accurate taxonomic identification and binning of short sequences (Qin et al. 2010; Kunin et al. 2010; Haas et al. 2011; Rodrigue et al. 2010).

[2] Alpha diversity is a summary measure of intra-sample variability, which captures both the organismal richness of the sample and the evenness of the organisms' abundance distribution.

using the vegan (Oksanen et al., 2011) and picante (Kembel et al., 2010) packages, and/or JMP Pro 12 software. To compare between-sample variations in the composition of the total microbial community, unweighted and weighted UniFrac distances (Lozupone and Knight, 2005) were calculated with the QIIME pipeline (Caporaso et al., 2010). PCoA (Principal Coordinate Analysis) was performed on both distance matrices and coordinates were used to draw 3D graphical outputs.

For analysis, the age variable was used both as a continuous factor by month and a grouped-age variable (0-3 mo., 4-7 mo., 8-11 mo., and 12+ mo.). These age groups were determined based on common life stages that occur around chronological transitions appropriate for this population. Infants 0-3 mo. largely received milk substrates, whereas mothers in this area tend to introduce "to taste" complementary foods around the 4th month of life. Thus, the 4-7 mo. group captured the transition to complementary feeding. 8-11 group is a transitional group where infants are often still receiving milk



Summary Statistics on Birth Mode and Early feeding by Age

Age	Breast Fed	Mixed Fed	Formula Fed
0-3 mo.			
VD (n=28)	12	14	2
CD (n=8)	5	3	0
4-7 mo.			
VD (n=39)	18	17	4
CD (n=15)	3	9	3
8-11 mo.			
VD (n=33)	12	14	7
CD (n=11)	6	3	2
12+ mo.			
VD (n=38)	17	13	8
CD (n=12)	5	5	2

Table 5: Summary statistics of birth mode and early feeding by age.

(either formula or breast), and 12+ mo. is commonly a time when infants will be weaned off of breast milk or bottle. Although the age group schema worked as expected, age was used as a continuous variable when appropriate.

The early feeding variable was originally a five-level scale: exclusively formula-fed; predominantly formula-fed; mixed-fed; predominantly breastfed; exclusively breastfed. For these analyses this five-level scale was reduced to three levels: exclusively formula-fed; mixed-fed; and exclusively breastfed. The two "predominant" feeding were merged into the mixed-fed group.

Results

H0: *A-diversity will increase steadily with age, a shift that will be associated with different bacterial assemblages at each age group.*

A-diversity increased significantly and steadily with age (F=60, P=.0001, R^2=.26) (see Figure 3), indicating the developmental progression towards a more complex and less heterogeneous

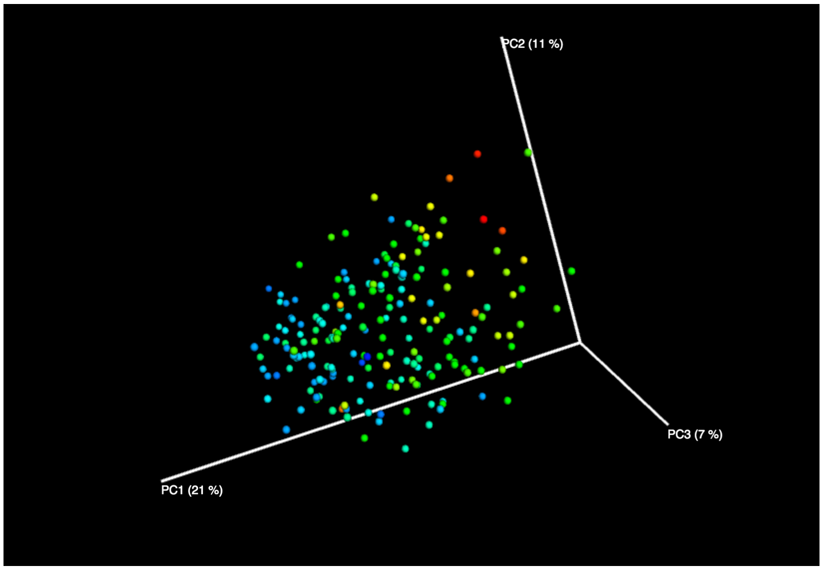


Figure 2: (PCoA) plot of age in months by microbial community composition.

principal coordinate analysis (PCoA) using unweighted UniFrac distance (Lozupone and Knight 2005) based on the MetaOTUs showed that the samples most clearly clustered according to age in months. Deepest blues represent month 0 and the darkest reds represent month 22.

community. Principal coordinate analysis (PCoA; unweighted UniFrac distance) (Lozupone and Knight 2005) based on the MetaOTUs confirmed that the samples most clearly clustered according to age in months (Figure 2).

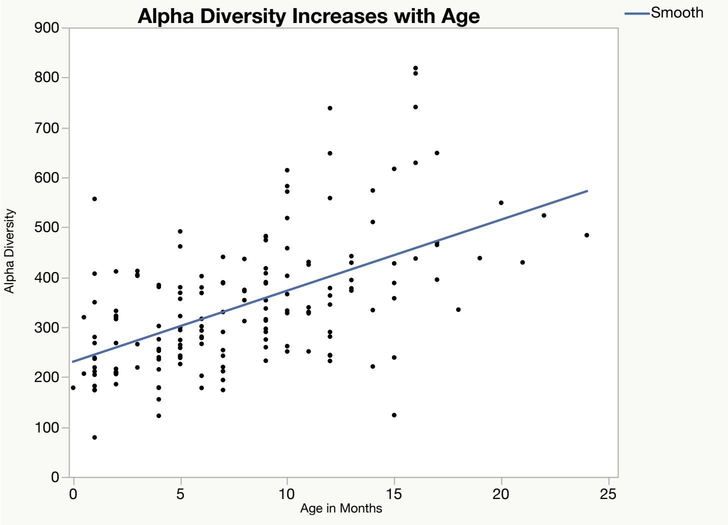


Figure 3: A-diversity steadily increases with age.

(F=60, P=.0001, R^2=.26).

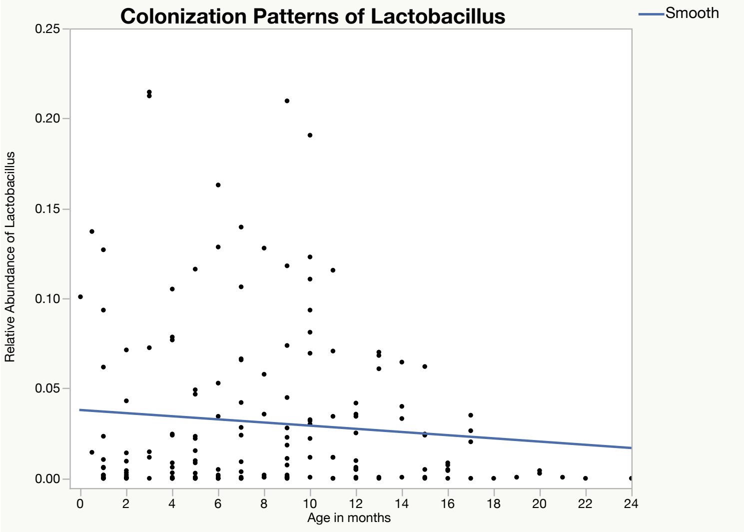


Figure 4: Colonization patterns of *Lactobacillus*.

***Lactobacillus* is an early colonizer, and decreases in abundance after the first year. This population of infants harbor considerably lower levels of this bacterium than other studies.**

Relative abundance was then assessed to explore the global changes in taxa over time, and which microbes predicted the changes in α-diversity. Unsurprisingly, Firmicutes were the most abundant phyla in the general population of infants, and only increased relative abundance over time (see Figure 5). This phylum is generally represented by anaerobic *Streptococcus* and facultatively aerobic *Veillonella* genera. In contrast, the second most abundant phylum, Proteobacteria (represented by Enterobacteriaceae), decreased steeply over time.

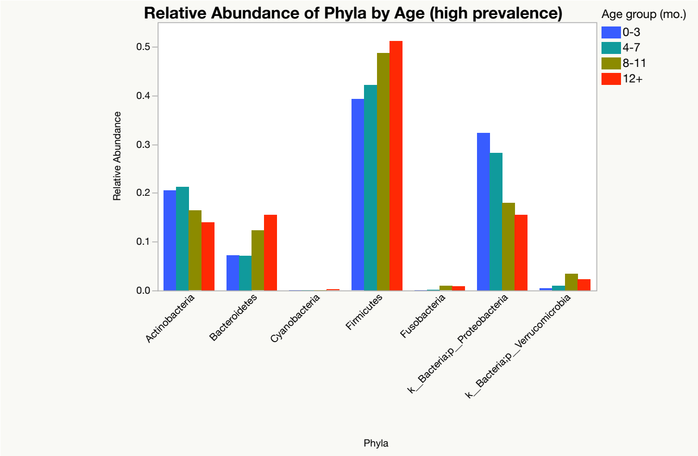


Figure 5: Relative abundance of the most common 7 phyla.

Firmicutes are the most common phylum, increasing relative abundance over time. Actinobacteria and Proteobacteria decrease over the course of the first year, while Bacteroidetes and Verrucomicrobia increase slightly over time.

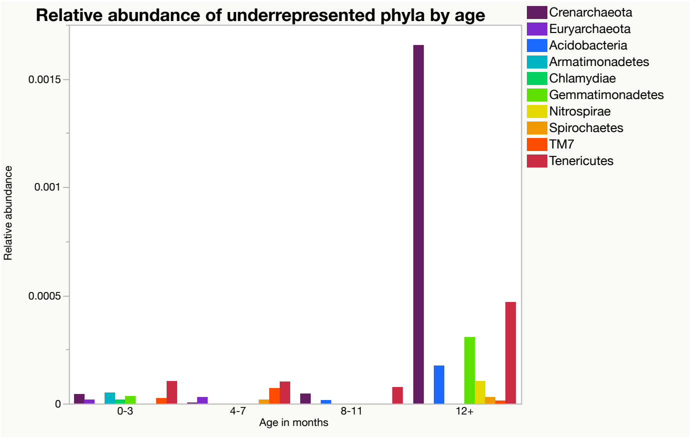


Figure 6: Relative abundance of less common phyla.

Crenarchaeota are the most abundant in the 12+ age group. Tenericutes increase with age, although relative abundance remains low until past the first year of life.

Actinobacteria (predominantly Bifidobacterium) also steadily decreased by the end of the first year. In these later months, Bacteroidetes increased abundance. Verrucomicrobia and Fusobacteria were underrepresented in 0-3 mo. age range but steadily increased in abundance over time, while Crenarchaeota (Archaea) and Cyanobacteria only appeared after the first year (see Figure 5). Less commonly represented phyla can be seen in Figure 6, although little is known about the functional significance of these bacteria, particularly in the infact gut.

Similar to a low-income population of South Indian infants, the most prominent genera found in the infant gut were from the family Enterobacteriaceae (Kabeerdoss et al. 2013; Adlerberth et al. 1991). Although representative species from this family are understood to be some of the first colonizers of the infant gut (Favier et al. 2002), the length of time these bacteria are dominant in the Salvadoran sample is uncommon. Other prevalent genera in order of dominance were *Bifidobacterium, Bacteroides, Streptococcus, Megasphaera, Veillonella, and* Lachnospiraceae. Consistent with the literature, the genera *Bifidobacterium*, *Lactobacillus*, and *Veillonella* were identified as members of the 0-11 month microbiome, which indicated reduced oxygen concentration and increased production and utilization of lactic acid for a milk-heavy diet (Fredrik Bäckhed et al. 2015). Notably, levels of *Lactobacillus* were substantially lower in this population than reported in other populations, a status that was particularly sensitive to early feeding practices (Figure 4).

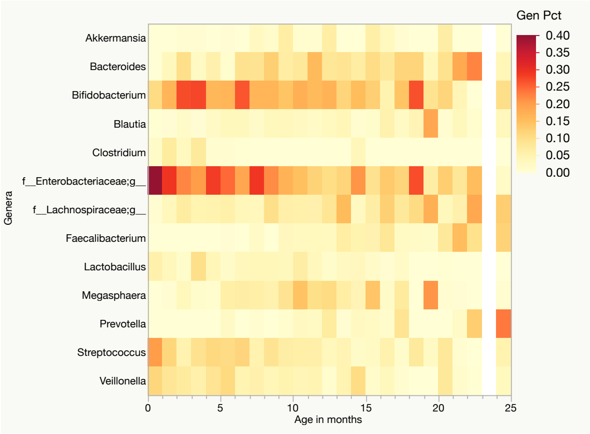


Figure 7: A heat map of bacterial colonization patterns over time.

Community members at 12 months included bacteria found in newborns, i.e., *Bacteroides, Bifidobacterium,* Lachnospiraceae*, Megasphaera, Streptococcus,* and *Veillonella*; bacteria emerging at 4 months, i.e., *Akkermansia, Blautia,* and *Faecalibacterium*; and bacteria that only occurred after 8 months, i.e., *Prevotella* and *Cetobacterium, Enterococcus* and *Sarcina*. *Lactobacillus* and *Streptococcus* were more prevalent in early months but diminished by 62-64% by the 12th month (see Figure 7). The bacteria that colonize the infant gut in later months were efficient degraders of dietary fibers and producers of short chain fatty acids (SCFAs), suggesting a microbial shift toward a more adult-like intestinal environment associated with the increased functional capacity for carbohydrate degradation. Thus, α-diversity increased over time, a shift that was driven by changes in the community composition and relative abundances of bacteria, providing evidence for a successional pattern of development (Figure 7).

H1a: *Alpha diversity should be higher in cesarean delivered compared to vaginally delivered infants at all ages.*

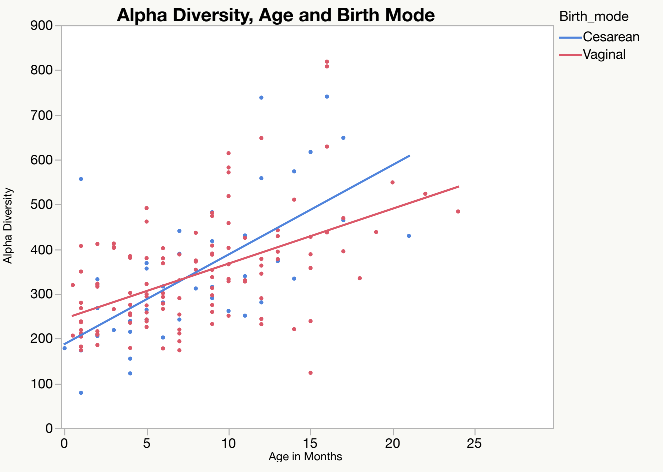
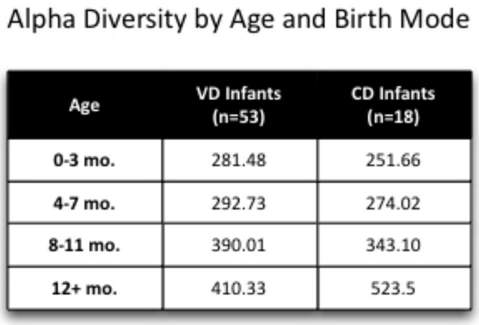


Figure 8: A-diversity, age and birth mode.

Vaginally Delivered (VD) infants on average start with higher α-diversity than do Cesarean Delivered (CD) infants.



Alpha Diversity by Age and Birth Mode

Age	VD Infants (n=53)	CD Infants (n=18)
0-3 mo.	281.48	251.66
4-7 mo.	292.73	274.02
8-11 mo.	390.01	343.10
12+ mo.	410.33	523.5

Table 6: Alpha diversity by age and birth mode.

VD infants have higher α-diversity until 12+ months when they have significantly lower levels than CD infants (F=3.9, P=.05).

Contrary to much of the literature, α-diversity is similar among the birth modes before controlling for age (Dominguez-Bello et al. 2010; Bäckhed et al. 2015). There was no significant difference found in α-diversity until ages >1 year, when VD infants had marginally lower levels of alpha diversity (F=3.9, P=.05). Although cesarean deliveries did not significantly affect infant microbial diversity until 12+ months, VD infants surprisingly, had a higher average α-diversity previous to this age (see Table 6). This suggests that mode of delivery may affect the developmental trajectories of infants in this study by influencing the rate at which infants acquire α-diversity over time. Although no significant relationship was found in the early months, VD infants began with higher average diversity and yet ended with lower overall diversity by 12+ mo., than CD infants (Figure 8). One study suggested that the intestinal microbiota of neonates delivered by cesarean section appeared to be less diverse, than the microbiota of vaginally delivered infants (Biasucci et al. 2008), although comparisons must be interpreted with caution as this study was a cross-sectional study of 4 day-old neonates. In general, the reason for CD infants beginning with lower microbial diversity in the early months is unclear. Figure 8 does not describe clear patterns among the groups over time, although it does signal extreme outliers from both birth modes.

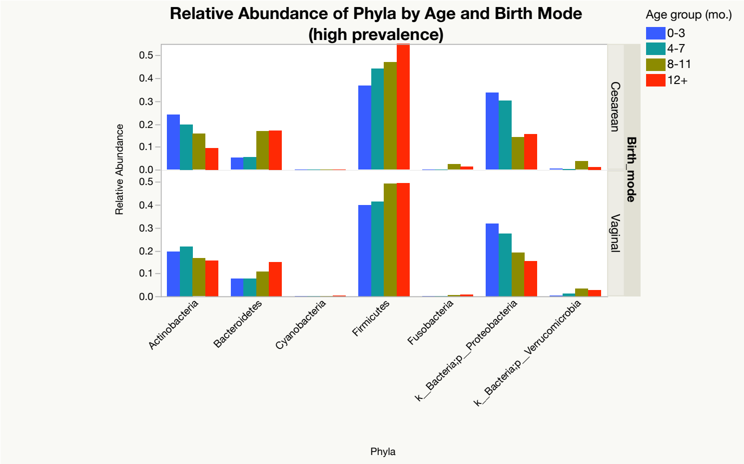
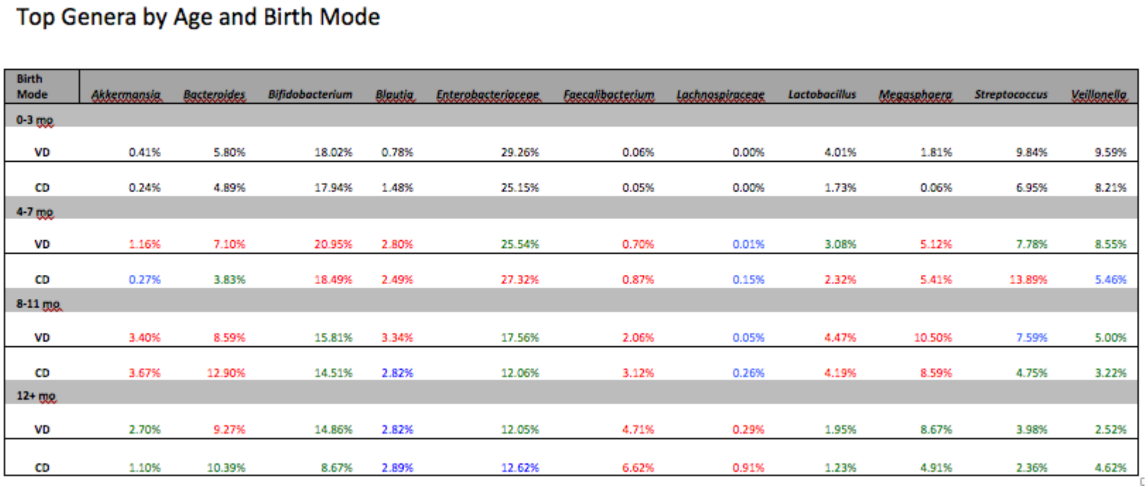


Figure 9: Relative abundance of the most common 7 phyla among both birth modes.

Firmicutes are the most common phylum, increasing relative abundance over time. Actinobacteria and Proteobacteria decrease over the course of the first year, while Bacteroidetes and Verrucomicrobia increase slightly over time.

H1b: *Vaginally delivered infants should reflect a vertically inherited microbiota including higher levels of Bifidobacterium, Lactobacillus, and Streptococcus. Cesarean delivered infants will have a more horizontal exposure reflected in their microbiota, including skin and environmental microbes including Corynebacterium, Dermacoccus*, and *Enhydrobacter*.

To discover signature taxa of vaginally delivered (VD) and Cesarean section-delivered (CD) infants, microbial community composition was analyzed.



Top Genera by Age and Birth Mode

Birth Mode	*Akkermansia*	*Bacteroides*	*Bifidobacterium*	*Blautia*	*Enterobacteriaceae*	*Faecalibacterium*	*Lachnospiraceae*	*Lactobacillus*	*Megasphaera*	*Streptococcus*	*Veillonella*
0-3 mo											
VD	0.41%	5.80%	18.02%	0.78%	29.26%	0.06%	0.00%	4.01%	1.81%	9.84%	9.59%
CD	0.24%	4.89%	17.94%	1.48%	25.15%	0.05%	0.00%	1.73%	0.06%	6.95%	8.21%
4-7 mo											
VD	1.16%	7.10%	20.95%	2.80%	25.54%	0.70%	0.01%	3.08%	5.12%	7.78%	8.55%
CD	0.27%	3.83%	18.49%	2.49%	27.32%	0.87%	0.15%	2.32%	5.41%	13.89%	5.46%
8-11 mo											
VD	3.40%	8.59%	15.81%	3.34%	17.56%	2.06%	0.05%	4.47%	10.50%	7.59%	5.00%
CD	3.67%	12.90%	14.51%	2.82%	12.06%	3.12%	0.26%	4.19%	8.59%	4.75%	3.22%
12+ mo											
VD	2.70%	9.27%	14.86%	2.82%	12.05%	4.71%	0.29%	1.95%	8.67%	3.98%	2.52%
CD	1.10%	10.39%	8.67%	2.89%	12.62%	6.62%	0.91%	1.23%	4.91%	2.36%	4.62%

Table 7: Relative abundance of top genera in infants of different age groups and birth modes.

VD = Vaginally Delivery, CD = Cesarean Delivery. Red represents an increased proportion from the prior age group, green represents a decline, and blue signals stability.

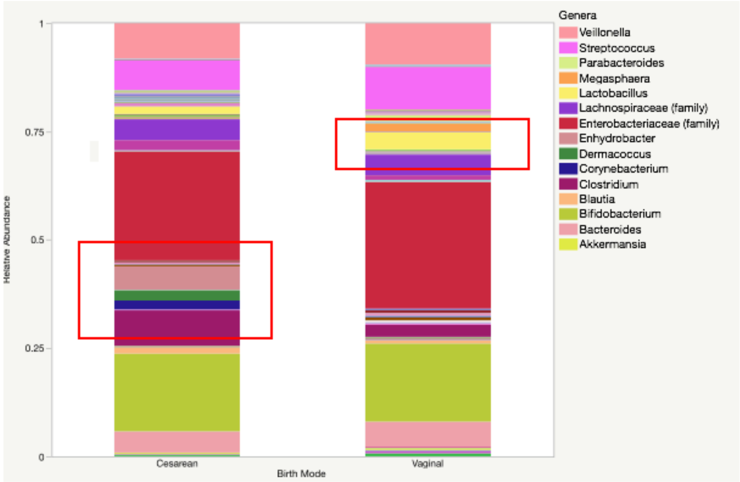


Figure 10: 100% stacked bar graph of bacterial taxonomic composition among infants age 0-3 mo.

The highlighted box shows that cesarean delivered infants harbored Enhydrobacter, Dermacoccus and Corynebacterium and Clostridium, bacteria associated with skin and inflammation (see red rectangle). Vaginally delivered infants harbored increased amounts of Lactobacillus and Megasphaera.

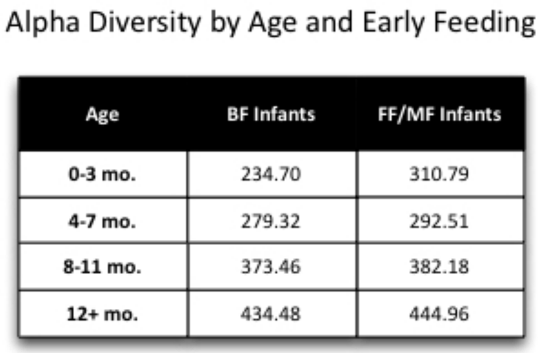
CD infants appeared to differ in certain respects from the VD infants in the community composition of phyla and genera over time. In the first 3 months of life, the VD group harbored proportionally higher levels of Firmicutes and Bacteroidetes, lower levels of Actinobacteria, similar levels of Proteobacteria and a small proportion of Verrucomicrobia (exclusively found in VD infants), than the CD infants of the same age (Figure 9).

At the genus level, both birth modes harbored high levels of microbes from the Enterobacteriaceae family (29.3% in VD infants and 25.1% in CD infants) at ages (0-3 months). Infants in this age group harbored similarly high levels of *Bifidobacteria*, *Bacteroides*, and *Veillonella* (see Table 7). VD infants harbored higher levels of *Lactobacillus* (VD=4%, CD=1.7%) and *Streptococcus* (9.8%, 6.9%). Alternatively, CD infants had significantly higher levels of *Clostridium* (8.1%) compared to their vaginally birthed counterparts (2.4%) (F=8.7, P=.007). Other bacteria such as *Corynebacterium*, *Dermacoccus*, *Enhydrobacter*, and *Erysipelotrichaceae* were found only in CD infant microbiomes, suggesting increased exposure to environment and human skin (see Figure 10). Interestingly, *Megasphaera* and *Parabacteroides* were exclusive genera to vaginally birthed infants in the first three months, indicating an early capacity for milk metabolism (Table 7).

At 4-7 months, levels of Enterobacteriaceae and *Bifidobacterium* remained high in both birth modes (Table 7). VD children harbored higher levels of *Bacteroides* (7.1%, 3.8%), and *Veillonella* (8.6%, 5.5%), however CD infants overtook their fellow VD infants in levels of Enterobacteriaceae*,* Lachnospiraceae and *Streptococcus* in this age group, showing an increasingly pro-inflammatory microbiota. Levels of *Lactobacillus* evened out among the birth modes, although they remained low at 2.9% and 2.3% for VD and CD infants. *Akkermansia* first appeared in this age group in VD infants only, and CD infants nearly doubled levels of *Streptococcus* from the prior age group (Table 7).

By 8-11 mo, levels of Enterobacteriaceae fell in both birth modes, most notably in CD infants (17.6%, 12.1%). Levels of *Bifidobacterium* also decreased along with levels of *Megasphaera* and *Streptococcus*. CD infants acquired increased levels of *Bacteroides* and Lachnospiraceae (Table 7).

By 12 + mo. both VD and CD children had greater richness and evenness in their microbial gut communities compared to younger age groups, giving no one species more than 15% of the overall composition. At this age, *Streptococcus* had receded to a small portion of the bacterial community, while Lachnospiraceae and *Prevotella* become increasingly present community constituents among both groups. The key differences at this age range was that VD infants had higher levels of *Akkermansia* (2.7%, 1.1%) (although not statistically significant) and *Megasphaera* (8.7%, 4.9%) (F=5.8, P=.02). In summary, there did appear to be some differences among VD and CD infants over time, although the differences were often not statistically significant. Whether levels of difference detected by statistical analyses equate to functional significance is a question for future research, and will be explored more profoundly in chapters 4 and 5. Regardless of significance, the VD infants seemed to reflect a more vertically inherited microbiome, including the hypothesized bacteria *Bifidobacterium*, *Lactobacillus*, and *Streptococcus,* and bacteria associated with milk metabolism such as *Megasphaera*. Interestingly, even without controlling for age and despite the low counts in the overall population, vaginally delivered infants harbor significantly higher levels of Lactobacillus than cesarean delivered infants (F=5.4, P=.02). Meanwhile CD infants harbored higher levels of skin-associated microbes including *Corynebacterium*, *Dermacoccus*, *Enhydrobacter*, and *Erysipelotrichaceae,* and other pro-inflammatory bacteria including Clostridium.



Alpha Diversity by Age and Early Feeding

Age	BF Infants	FF/MF Infants
0-3 mo.	234.70	310.79
4-7 mo.	279.32	292.51
8-11 mo.	373.46	382.18
12+ mo.	434.48	444.96

Table 8: A-diversity by ages and early feeding.

Breastfed infants (BF) compared to formula and mixed fed infants have on average lower α-diversity.

H2a: *Alpha diversity should be higher in formula fed infants compared to breastfed infants.*

Next, the role of early feeding practices on the establishment of the gut microbiota during the first year of life was evaluated. Feeding seemed to affect both the α-diversity and the relative abundance of microbial composition over time. In the first 3 months of life, exclusively breastfed (BF) infants had significantly lower levels of α-diversity than formula fed (FF) and mixed fed (MF) infants (F=4.9, P=.016) (see Table 8 and Figure 12).

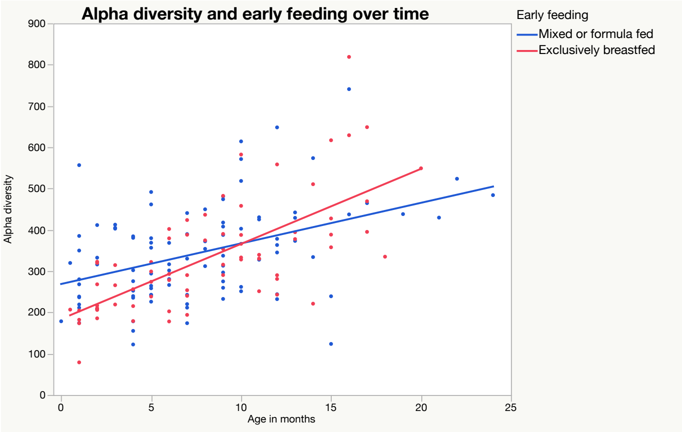


Figure 12: Early feeding and alpha diversity.

Breastfed infants begin with lower alpha diversity and on average have higher diversity than formula fed infants towards the end of the second year of life.

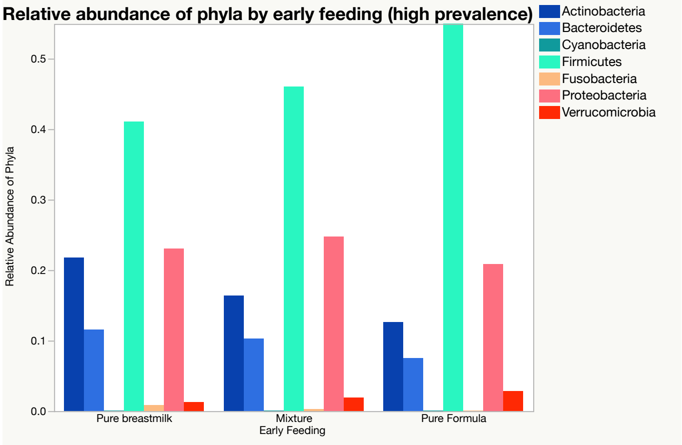


Figure 11: Phyla by early feeding.

Firmicutes are highest among the exclusively formula fed infants, while exclusively breastfed infants have higher Actinobacteria and Bacteroidetes.

Moreover, the differences in community composition between BF and FF infants are explored in the following section.

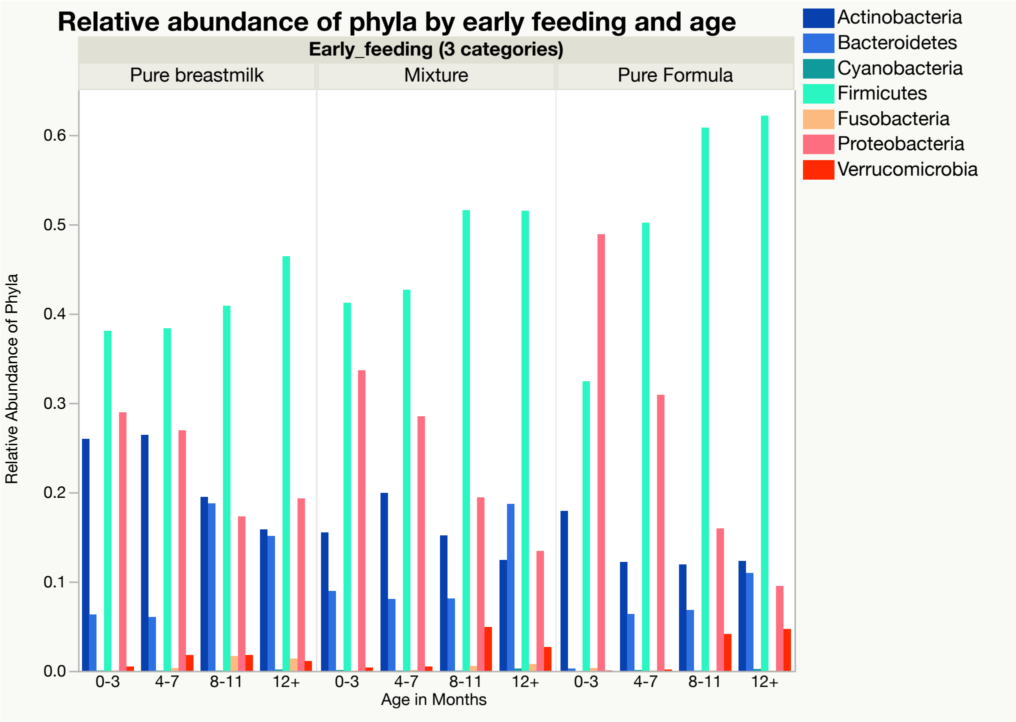


Figure 14: Phyla by early feeding and age group.

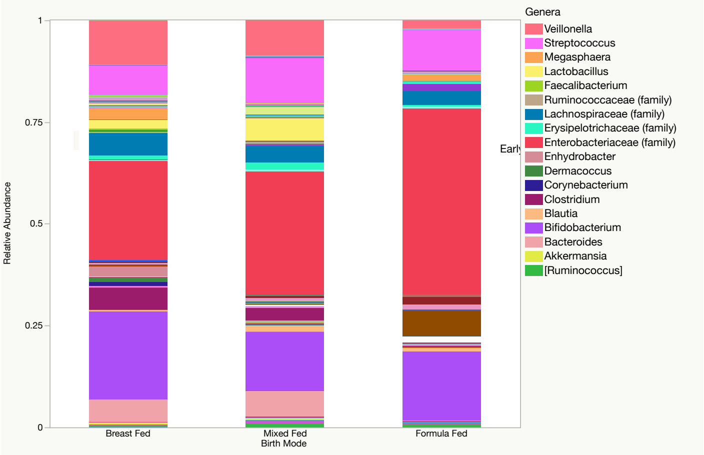


Figure 15: 100% stacked bar graph of bacterial taxonomic composition among feeding groups, age 0-3 mo.

Bacterial taxonomic composition of human infant fecal matter (age 0-3 mo). The graph shows the proportion of bacterial families and genera as inferred by PCR amplification and pyroseqencing of the 16S rRNA.

H2b: *Breastfed infants should reflect breast-milk-associated microbiota, including higher levels of Bifidobacterium, Lactobacillus, Megasphaera and Veillonella. Formula fed infants should harbor bacteria associated with cow's milk-based formula including Blautia, Lachnospiraceae, and Enterobacteriaceae.*

At the phylogenetic

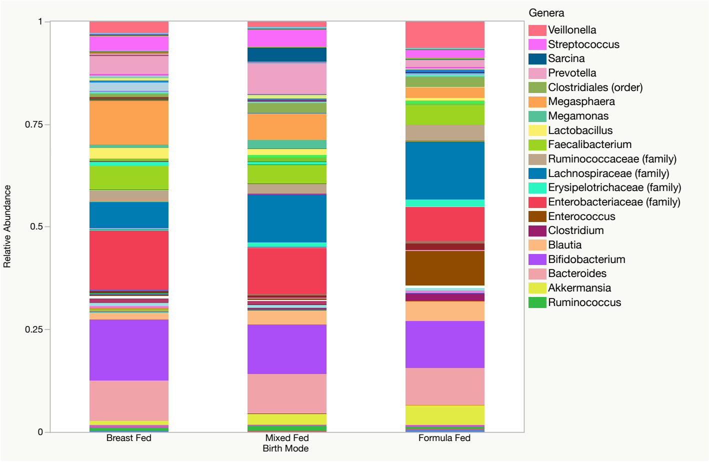
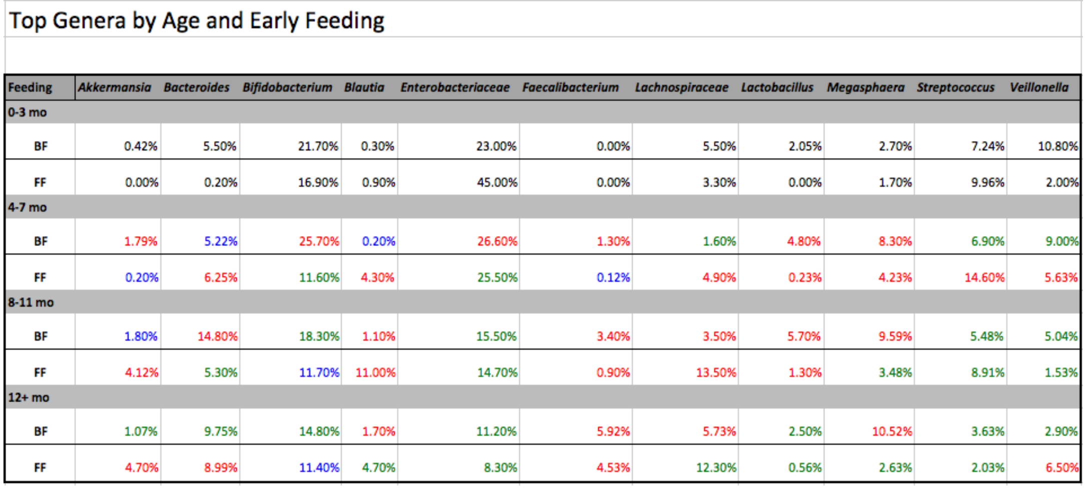


Figure 16: 100% stacked bar graph of bacterial taxonomic composition among feeding groups, age 12+ mo.

Bacterial taxonomic composition of human infant fecal matter (age 12+ mo). The graph shows the proportion of bacterial families and genera as inferred by PCR amplification and pyroseqencing of the 16S rRNA. Infants older than 1 yr. show increased diversity of genera, lower relative abundance of dominant taxa compared to infants 0-3 mo.



Top Genera by Age and Early Feeding

Feeding	*Akkermansia*	*Bacteroides*	*Bifidobacterium*	*Blautia*	*Enterobacteriaceae*	*Faecalibacterium*	*Lachnospiraceae*	*Lactobacillus*	*Megasphaera*	*Streptococcus*	*Veillonella*
0-3 mo											
BF	0.42%	5.50%	21.70%	0.30%	23.00%	0.00%	5.50%	2.05%	2.70%	7.24%	10.80%
FF	0.00%	0.20%	16.90%	0.90%	45.00%	0.00%	3.30%	0.00%	1.70%	9.96%	2.00%
4-7 mo											
BF	1.79%	5.22%	25.70%	0.20%	26.60%	1.30%	1.60%	4.80%	8.30%	6.90%	9.00%
FF	0.20%	6.25%	11.60%	4.30%	25.50%	0.12%	4.90%	0.23%	4.23%	14.60%	5.63%
8-11 mo											
BF	1.80%	14.80%	18.30%	1.10%	15.50%	3.40%	3.50%	5.70%	9.59%	5.48%	5.04%
FF	4.12%	5.30%	11.70%	11.00%	14.70%	0.90%	13.50%	1.30%	3.48%	8.91%	1.53%
12+ mo											
BF	1.07%	9.75%	14.80%	1.70%	11.20%	5.92%	5.73%	2.50%	10.52%	3.63%	2.90%
FF	4.70%	8.99%	11.40%	4.70%	8.30%	4.53%	12.30%	0.56%	2.63%	2.03%	6.50%

Table 9: Relative abundance of top genera in infants of different age groups by early feeding practices.

BF= Breastfed, FF= Formula Fed. Red represents an increased proportion from the prior age group, green represents a decline, and blue signals stability.

level, breastfed (BF) infants had lower Firmicutes and Verrucomicrobia than mixed fed (MF) or formula fed (FF) infants, and higher levels of Actinobacteria, Bacteroidetes, and Proteobacteria. For all three groups, Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, and increased in Firmicutes over time, decreased in Actinobacteria and Proteobacteria, and fluctuated in Bacteroidetes, Fusobacteria and

Verrucomicrobia over time. Notably, at 0-3 mo. FF infants harbored higher Proteobacteria than Firmicutes, which decreased dramatically as levels of

Firmicutes steadily increased (Figure 14). There was a general increase in Bacteriodetes and a substantial increase of Verrucomicrobia between 8-11 and 12+ months (Figure 11 and Figure 14).

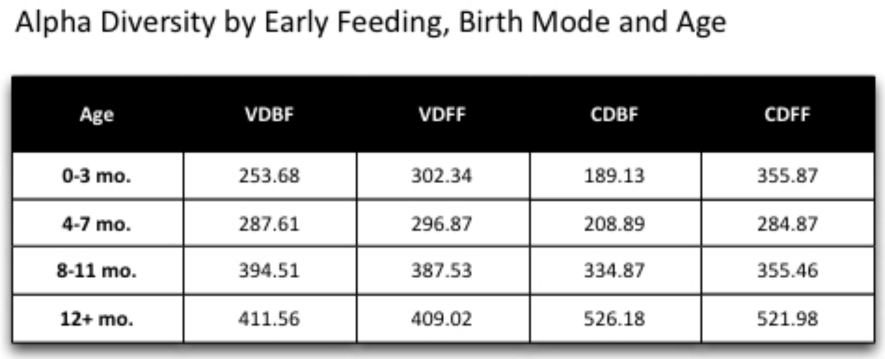
In the early months (0-3 mo.), breastfed infants harbored substantially higher levels of *Bifidobacterium*, *Veillonella,* and *Staphylococcus,* and only half of the levels of Enterobacteriaceae compared to FF infants (Table 9), although small sample size prevented statistical analyses to calculate significance. While BF infants contained only 2.05% *Lactobacillus*, FF infants had 0% in this age range (Table 9).

At 4-7 mo. both early feeding groups had similar levels of Enterobacteriaceae. BF infants continued to harbor more than double the level of *Bifidobacterium* compared to FF infants. *Lactobacillus* levels also increased to 4.8% in BF infants, but remained nearly undetectable in FF infants (0.3%). *Streptococcus* levels were doubled in FF infants compared to BF infants (14.6%, 6.9%). *Akkermansia* first appeared in BF infants, but not in FF counterparts, while *Blautia* was found exclusively in FF infants (Table 9 and Figure 15).

At 8-11 months, BF infants had significantly higher levels of *Bifidobacterium* (F=3.8, P=.03), *Bacteroides* (F=4.0, P=.026) and Lactobacillus (F=6.6, P=.01) than FF infants. Levels of Enterobacteriaceae decreased in both feeding groups, while FF experienced significant increases in *Blautia* (F=9.3, P=.0005) compared to BF infants, and compared to the FF 4-7 age group (F=4.2, P=.02). FF infants also harbored significantly higher Lachnospiraceae (F=7.7, P=.0015), and Ruminococcus (F=6.7, P=.0028) than BF infants (Table 9).

At 12+ mo. FF infants had significantly higher levels of *Blautia* (F=3.6, P=.036), Lachnospiraceae (F=4.2, P=.02) and *Veillonella* (F=3.5, P=.039). BF infants had higher *Lactobacillus* (F=3.4, P=.04), and *Megasphaera* (F=3.2, P=.04). Critically, the cessation of breast-feeding had profound effects on the microbiota in 12-month-old infants and shifted the microbial ecology toward an adult-like composition (see Figure 16 and Figure 19), enriched in Lachnospiraceae (F=5.8, P=.02)*, Enterococcus* (F=4.2%, P=.04), *Akkermansia* (F=5.8, P=.02)*, Veillonella* (F=5, P=.03)*,* and *Blautia* (F=4.6, P=.03). In contrast, the gut microbiome of infants breast-fed at 12 months was still dominated by Bifidobacterium (14.8%), Enterobacteriaceae (11.2%)*, Bacteroides* (9.75%)*, Lactobacillus* (2.5%), and *Megasphaera* (10.5%), bacteria that are associated with breast milk (Jost et al. 2014). (Table 9 and Figure 16),

Overall, *Bifidobacterium, Lactobacillus, Megasphaera, Staphylococcus,* and *Veillonella* were positively associated with breastfeeding, while *Blautia, Clostridiales, Enterobacteriaceae, Enterococcus, Lachnospiraceae, Ruminococcus, and Streptococcus* were associated with formula feeding. In particular, Enterobacteriaceae and *Blautia* were associated with a powdered cow's milk instead as alternative to formula. FF infants experienced delayed colonization of *Akkermansia*, *Bacteroides*, and *Faecalibacterium*, and harbored consistently higher levels of inflammatory bacteria including *Blautia*, Lachnospiraceae and *Streptococcus* and at 0-3 months FF infants harbored double the levels of Enterobacteriaceae compared to BF infants. There was also a significant difference between BF and MF infants (P=.04), suggesting that even a partial diet of cow's milk formula is enough to promote the early proliferation of this family of bacteria. BF infants harbored higher levels of *Bifidobacterium* and *Lactobacillus* at all age groups, indicating that these bacteria are highly dependent on early feeding practices.



Alpha Diversity by Early Feeding, Birth Mode and Age

Age	VDBF	VDFF	CDBF	CDFF
0-3 mo.	253.68	302.34	189.13	355.87
4-7 mo.	287.61	296.87	208.89	284.87
8-11 mo.	394.51	387.53	334.87	355.46
12+ mo.	411.56	409.02	526.18	521.98

Figure 17: A-diversity by early feeding, birth mode and age.

Breastfed infants of both birth modes steadily increase in α-diversity levels, while formula fed infants of both birth modes start with high α-diversity, decrease slightly in the 4-7 mo. and increase again after 8+ mo.

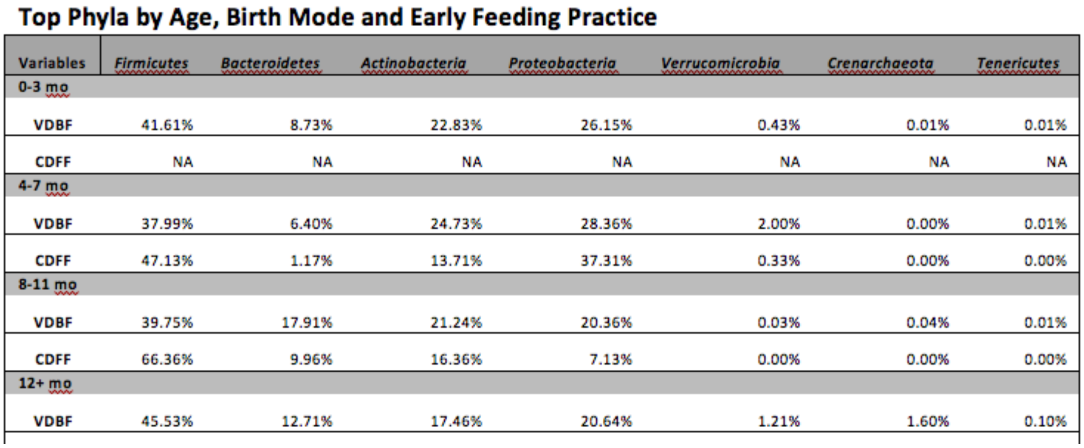
H3: *Birth-mode and early feeding interact as synergistic factors, either contributing to enhanced levels of anti-inflammatory, age-appropriate and breast-milk associated microbes, or together increasing dysbiosis, instability, delayed colonization and increased levels of pro-inflammatory microbiota.*

To evaluate the interactions of birth mode and early feeding variables on the establishment of the gut microbiota during the first year of life, these factors were combined into sub-groups; the relative abundance of bacteria in vaginally delivered and breast-fed (VDBF) infants was examined, followed by the influence of Cesarean delivered and formula-fed (CDFF) infants. The following sections break down **H3** hypothesis into smaller, testable hypotheses.

H3a: *A-diversity should be lowest in the VDBF infants at 0-3 mo., increasing steadily over time, becoming the most diverse sub-group by 12+ mo.*

VDBF and CDBF Infants have the lowest levels of α-diversity at 0-3 months, compared to VDFF and CDFF infants (see Figure 17). Regardless of delivery mode, BF infants increase α-diversity steadily over time, whereas FF infants of both birth modes had volatile diversity patterns (Figure 17). This suggests that breastfeeding contributes to a more stable development in the early months of the first year irrelevant of birth mode. However, interestingly CDBF and CDFF infants have the highest levels of diversity at the end of the first year. It is unclear why birth mode seems to show an effect at the end of the first year despite milk-feeding practices in this time, although breast-feeding is likely instrumental in supporting a stable increase in diversity over the first year.

H3b: *Vaginally delivered, breastfed (VDBF) infants should harbor a vertically-inherited microbiota, replete with anti-inflammatory, age-appropriate and breast-milk degrading bacteria, including higher levels of Bifidobacterium, Lactobacillus, Megasphaera and Veillonella.*



Top Phyla by Age, Birth Mode and Early Feeding Practice

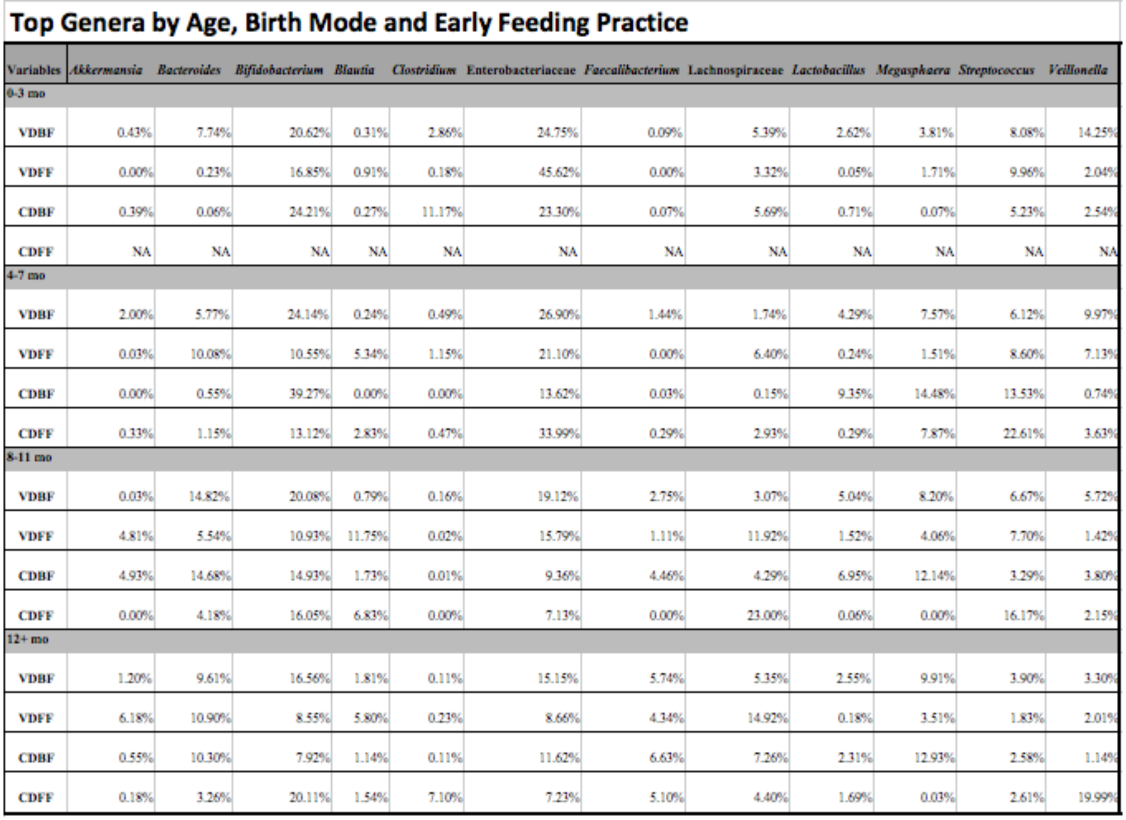
Variables	*Firmicutes*	*Bacteroidetes*	*Actinobacteria*	*Proteobacteria*	*Verrucomicrobia*	*Crenarchaeota*	*Tenericutes*
0-3 mo							
VDBF	41.61%	8.73%	22.83%	26.15%	0.43%	0.01%	0.01%
CDFF	NA	NA	NA	NA	NA	NA	NA
4-7 mo							
VDBF	37.99%	6.40%	24.73%	28.36%	2.00%	0.00%	0.01%
CDFF	47.13%	1.17%	13.71%	37.31%	0.33%	0.00%	0.00%
8-11 mo							
VDBF	39.75%	17.91%	21.24%	20.36%	0.03%	0.04%	0.01%
CDFF	66.36%	9.96%	16.36%	7.13%	0.00%	0.00%	0.00%
12+ mo							
VDBF	45.53%	12.71%	17.46%	20.64%	1.21%	1.60%	0.10%

Table 10: Relative abundance of top Phyla in infants of different age groups by birth mode and early feeding practices.

VDBF= Vaginally Delivered & Breastfed and CDFF= Cesarean Delivered & Formula Fed.

Firmicutes (41.6%) was the most abundant phylum among VDBF infants at 0-3 months. This group of infants experienced a gradual increase after 12 months (47.4%). The next most abundant phylum in this sub-group was Proteobacteria (26.2%), which decreased slightly in abundance over time to hover around 20% by the second year of life. Actinobacteria was the next most abundant phylum at 22.8% decreasing over time to 17.5%. Bacteroidetes were at 8.7% in the first months, and fluctuated over time, reaching heights of 17.9%, and ending at 12.9% by the end of the first year (see Table 10).

At the genus level, VDBF infants at 0-3 months have high levels of Enterobacteriaceae*, Bifidobacterium, Veillonella, Streptococcus,* and *Bacteroides* in descending order of predominance. By 4-7 mo. these infants have increased



Top Genera by Age, Birth Mode and Early Feeding Practice

Variables	*Akkermansia*	*Bacteroides*	*Bifidobacterium*	*Blautia*	*Clostridium*	Enterobacteriaceae	*Faecalibacterium*	Lachnospiraceae	*Lactobacillus*	*Megasphaera*	*Streptococcus*	*Veillonella*
0-3 mo												
VDBF	0.43%	7.74%	20.62%	0.31%	2.86%	24.75%	0.09%	5.39%	2.62%	3.81%	8.08%	14.25%
VDFF	0.00%	0.23%	16.85%	0.91%	0.18%	45.62%	0.00%	3.32%	0.05%	1.71%	9.96%	2.04%
CDBF	0.39%	0.06%	24.21%	0.27%	11.17%	23.30%	0.07%	5.69%	0.71%	0.07%	5.23%	2.54%
CDFF	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-7 mo												
VDBF	2.00%	5.77%	24.14%	0.24%	0.49%	26.90%	1.44%	1.74%	4.29%	7.57%	6.12%	9.97%
VDFF	0.03%	10.08%	10.55%	5.34%	1.15%	21.10%	0.00%	6.40%	0.24%	1.51%	8.60%	7.13%
CDBF	0.00%	0.55%	39.27%	0.00%	0.00%	13.62%	0.03%	0.15%	9.35%	14.48%	13.53%	0.74%
CDFF	0.33%	1.15%	13.12%	2.83%	0.47%	33.99%	0.29%	2.93%	0.29%	7.87%	22.61%	3.63%
8-11 mo												
VDBF	0.03%	14.82%	20.08%	0.79%	0.16%	19.12%	2.75%	3.07%	5.04%	8.20%	6.67%	5.72%
VDFF	4.81%	5.54%	10.93%	11.75%	0.02%	15.79%	1.11%	11.92%	1.52%	4.06%	7.70%	1.42%
CDBF	4.93%	14.68%	14.93%	1.73%	0.01%	9.36%	4.46%	4.29%	6.95%	12.14%	3.29%	3.80%
CDFF	0.00%	4.18%	16.05%	6.83%	0.00%	7.13%	0.00%	23.00%	0.06%	0.00%	16.17%	2.15%
12+ mo												
VDBF	1.20%	9.61%	16.56%	1.81%	0.11%	15.15%	5.74%	5.35%	2.55%	9.91%	3.90%	3.30%
VDFF	6.18%	10.90%	8.55%	5.80%	0.23%	8.66%	4.34%	14.92%	0.18%	3.51%	1.83%	2.01%
CDBF	0.55%	10.30%	7.92%	1.14%	0.11%	11.62%	6.63%	7.26%	2.31%	12.93%	2.58%	1.14%
CDFF	0.18%	3.26%	20.11%	1.54%	7.10%	7.23%	5.10%	4.40%	1.69%	0.03%	2.61%	19.99%

Table 11: Relative abundance of top genera in infants of different age groups by birth mode and early feeding practices.

VDBF= Vaginally Delivered & Formula Fed, CDBF= Cesarean Delivered & Breastfed, CDFF= Cesarean Delivered & Formula Fed.

levels of *Akkermansia*, *Faecalibacterium*, *Lactobacillus*, and *Megasphaera* as compared to their previous age. Bacteria associated with an inflammatory state are decreasing, including Lachnospiraceae*, Streptococcus, and Clostridium.*

In the 8-11 month range, infants in the VDBF subgroup have relatively stable levels of *Bifidobacterium*, Lachnospiraceae, *Streptococcus*, *Lactobacillus*, and *Megasphaera*. Levels of *Bacteroides* and *Faecalibacterium* continue to increase while levels of Enterobacteriaceae, *Bifidobacterium*, *Veillonella* decline.

Predictably, the 12+ age group saw decreases in milk-fermenting microbes such as *Lactobacillus* and *Veillonella,* while bacteria from the family

Megasphaera, and genus Lachnospiraceae (both known producers of SCFAs) continued to augment. Bacteria that at age 0-3 mo. dominated the intestinal tract of VDBF infants showed a reduced relative abundance by 12+, likely making room for a more diverse, mature microbial community (see Table 11).

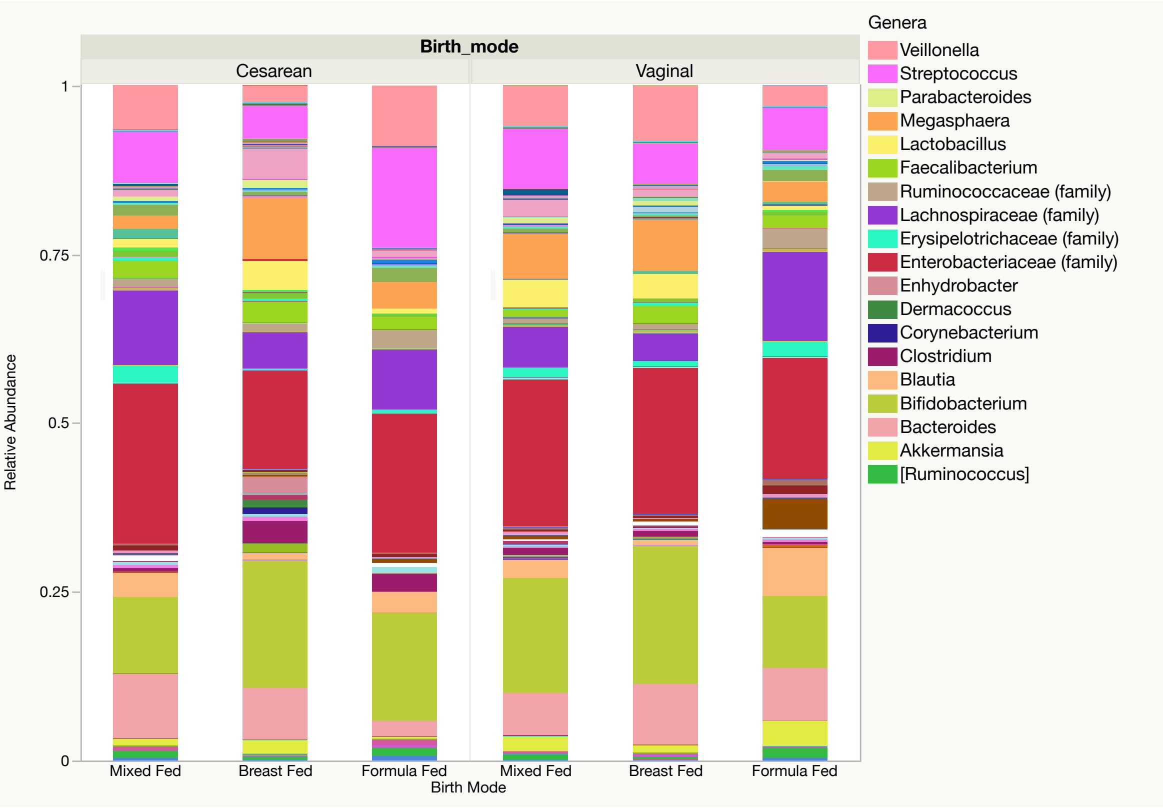


Figure 18: 100% stacked bar graph of bacterial taxonomic composition among birth modes and early feeding categories, age 0-24 mo.

H3c: *Cesarean Delivered, Formula fed (CDFF) infants should have dysbiotic, and unstable microbiomes that show delayed colonization patterns with increased levels of pro-inflammatory microbiota including Blautia, Lachnospiraceae, and Enterobacteriaceae.*

The sample size of CDFF infants at 0-3 months was too small to calculate levels of bacterial taxa (see Table 11). By 4-7 months, however, this subgroup of infants had the highest levels of Firmicutes (47.1%), which continued to increase over time. This age group also had very high Proteobacteria (37.3%), which went back down to 10% after month 8. Actinobacteria actually started very low (13.7%) and increased over time as opposed to all the other groups of infants. Bacteroidetes started and remained extremely low in this population (1.17%).

The most prevalent genera at 4-7 mo. in CDFF infants were Enterobacteriaceae (34%), *Streptococcus* (22.6%), *Bifidobacteria* (13%), and *Megaspharaea* (8%) (Table 11). Compared to VDBF infants in this age group, CDFF infants had higher levels of *Streptococcus* (22.61% compared to 6.12% VBBF), Enterobacteriaceae (CDFF=34%, VDBF=26.9%), Lachnospiraceae (CDFF=2.9 %, VDBF=1.7%), and *Blautia* (CDFF=2.8%, VDBF=.2%), which are pro-inflammatory bacteria, and lower levels of anti-inflammatory bacteria such as *Akkermansia* (CDFF=.3%, VDBF=2%), *Bifidobacterium* (CDFF=13%, VDBF=24%), *Faecalibacterium* (CDFF=.3%, VDBF=1.4%), and *Bacteroides* (CDFF=1.15%, VDBF=5.8%), bacteria that are expected to be increasing at this age.

At 8-11 months, CDFF infants experienced a 27% drop in Enterobacteriaceae, (levels that remained stable into the 12th month), and a 20% increase in Lachnospiraceae from the prior age group, still maintaining levels 20% higher than their VDBF counterparts of the same age category. Early blooms of Lachnospiraceae have been associated with obesity (Cho et al. 2012), most likely due to their short chain fatty acid (SCFA) production (Duncan et al. 2002). *Streptococcus* levels also remained high in this CDFF group (9.5% higher than their same age VDBF group). This subgroup also saw slowly augmenting proportions of *Bacteroides*, but levels would never reach those of their VDBF counterparts. These infants continued to have negligable amounts of other important anti-inflammatory microbes including *Akkermansia* (0%), *Faecalibacterium* (0%), *Lactobacillus* (.06%), and *Megasphaera* (0%) (Table 11).

At 12+ months CDFF infants harbored levels of *Bifidobacterium* and *Faecalibacterium* equivalent to those of VDBF infants at age 0-3 mo, indicating catch-up from a delayed colonization patterns. *Clostridium* levels increased in this age group (rising to 7.1% compared to .11% in the VDBF group). *Streptococcus* levels also decreased nearly 14% from the younger age group, reaching equivalent levels after the first year of life (Table 11).

Together, these analyses suggest that CDFF infants have less stable, more pro-inflammatory microbiomes than VDBF infants. This may be due to a delayed colonization pattern, was well as higher levels of pro-inflammatory microbiota including *Blautia, Clostridium,* Lachnospiraceae, Enterobacteriaceae and *Streptococcus*. Moreover, these infants had relatively lower levels of breast-milk associated *Bifidobacterium*, *Bacteroides, Lactobacillus*, and *Megasphaera* as well as anti-inflammatory *Akkermansia* and *Faecalibacterium* at key times during development (see Figure 18). These findings illustrate the relatively stable succession of different microbes in specific windows of time may be key to the early developmental patterns and functional outcomes of host-microbe interactions.

To statistically investigate if the combination of birth mode and early feeding affected the colonization of key microbes, standard multiple regression was performed. VDBF infants were significantly associated with increased levels of *Lactobacillus*, both before and after controlling for age (F= 3.7, P= .006), with an R^2 of .09. *Bifidobacteria* was also found to be significantly dependent upon the two factors (VDBF), with a regression equation of (F=4.5, P=.005), R2 = .08. *Streptococcus* was only associated with early feeding and age, (F=1.7, P=.006), R2=.14. As to be expected, *Blautia* was significantly associated with formula feeding (F=9.14, P=.0001), R2=.14, and Lachnospiraceae was associated with both factors (CDFF), (F=7.5, P=.0001), R=.12. *Akkermansia, Faecalibacterium,* and *Bacteroides* were only associated with age, although all three genera maintained higher and earlier levels in VDBF infants, highlighting the importance of a successional development.

Discussion

This chapter has explored the effect of age, birthing and feeding practices on the early development of intestinal microbiota in 71 Salvadoran infants over the first two years of life. Analyses confirmed that all three factors were significant drivers of the microbial assembly and maturation. There were nonrandom transitions in the infants' gut at age groups 0-3 mo., 4-7 mo., 8-11 mo., and 12+ mo., likely induced by the establishment of an anaerobic environment, nutrient availability, microbial interactions, and other environmental factors not captured in these variables, but explored in future chapters.

Results showed a significant and steady increase in α-diversity over time in all groups, pointing to the development of more complex and less heterogeneous microbiota over time, as reported previously in other populations of children (Fredrik Bäckhed et al. 2015; Yatsunenko et al. 2012). Consistent with other studies, results from the present study show that the maturation of the gut microbiota is a nonrandom process where distinct signature species and a network of changing positive and negative interactions between key microbial taxa can be identified at different ages (Koenig et al. 2010; Fredrik Bäckhed et al. 2015). The first step comprises early colonization by mainly Firmicutes (including anaerobic *Streptococcus* and facultatively aerobic *Veillonella*), Proteobacteria (lead by facultatively anaerobic bacteria including Enterobacteriaceae), and Actinobacteria (including anaerobic *Bifidobacteria*, especially in breastfed infants) (Rautava et al. 2012). The next succession step is the increase in the Bacteroidetes phylum of bacteria, as levels of Actinobacteria and Proteobacteria decrease. These findings were consistent with the idea that the length of breast-feeding and timing of weaning influences when infant microbiota converge towards an adult-like profile, possibly more than the introduction of complementary foods. Western populations (including the current study population) tend to wean infants within the first year, and thus the infant gut microbiome tends to mature around this time (Palmer et al. 2007; Koenig et al. 2010), whereas studies of non-western populations that breast feed into the third year of life show prolonged developmental periods that stretch the length of feeding period (Yatsunenko et al. 2012).

Beyond the general succession and development associated with age, this study showed that mode of delivery and feeding patterns have significant effects on gut microbiota assembly. Vaginally born infants were initially inoculated with microbes from the mother's birth canal, harboring significantly higher amounts of *Lactobacillus*. By one month of age, these infants harbored anti-inflammatory bacteria such as *Akkermansia,* whereas one-month-old infants who were delivered via C-section developed gut microbiota that more closely resembles skin, hospital and environmental microbes (Dominguez-Bello et al. 2010) including *Enhydrobacter*, *Clostridium*, *Corynebacterium*, and *Dermacoccus*. Surprisingly however, other predominant bacteria did not differ significantly among the delivery modes in the first months. Levels of *Bifidobacterium*, Enterobacteriaceae, Lachnospiraceae, and *Veillonella* were similar across birth modes in the first months, suggesting that delivery mode alone may not be as key of a factor compared to other social or ecological variables specific to this population.

Previous research has demonstrated that CD infants harbor initially more diverse, yet less stable microbiomes (Bäckhed et al. 2015, Rautava et al. 2012). Yet in this population, VD infants had a higher initial α-diversity than CD infants, a status that reversed by the end of the first year of life. Despite the significant difference in α-diversity by 12+ months, differences in relative abundance of microbes between the two birth modes became more difficult to detect as infants reached a year of age. These differences likely were less detectable because by this age the microbiome was more diverse, which for this population meant that no one species made up more than 15% of the relative abundance. Although community composition became more similar between the birth modes by 12 + months, CD infants had less consistent and more unstable colonization patterns, particularly among key bacterial genera compared to VD infants. VD infants harbored higher abundances of *Akkermansia, Bifidobacterium* and *Megasphaera* compared to CD infants, illustrating that birth mode may shape infant microbial development and function into the second year of life even when overall communities appear similar. These three microbes have been shown to improve digestion, gut health and maintenance, reduce inflammatory responses and enhance immunity through competitive exclusion (see chapter 5 for more details on microbial functions and interactions).

Reports in the literature conflict regarding the effect of diet on the composition of the infant GI microbiota. Several studies have reported that *Bifidobacteria* and *Lactobacillus* dominate the GI microbiota of breast-fed infants by several weeks of age (Stark and Lee 1982; Penders et al. 2006; Benno et al. 1984; Favier et al. 2002). These genera have generally been considered foundational bacteria that synergistically aid infant metabolism (Ward et al. 2006; Sela and Mills 2010), provide antimicrobial protection (Liévin et al. 2000), and contribute to intestinal development (Sjögren et al. 2009). Numerous studies have found a lower abundance of *Bifidobacteria* and a higher abundance of aerobic bacteria in the GI microbiota of formula-fed infants relative to breast-fed infants (Favier et al. 2002; Hall et al. 1990; Yoshioka et al. 1983; Harmsen et al. 2000; Balmer and Wharton 1989), yet other reports have found no such difference (Penders et al. 2005; Lundequist et al. 1985).

The current study found *Bifidobacteria* to be among the most dominant bacteria of both feeding groups, although levels were consistently higher in breastfed infants (ranging from 3.5% to 14% higher). Surprisingly, little to no prevalence of *Lactobacillus* was found in this population of infants. Breastfed infants ranged from 2%-5.7% relative abundance of *Lactobacillus*, whereas formula fed infants hovered between 0%-1.3%. It is difficult to discern whether this is a recent loss, or if this genus was never a numerically or functionally important member of the Salvadoran infant microbiome. Thus, it is unclear whether the present findings show a resilient, adaptive effort by the microbiome, an example of biodiversity and functional redundancy, or a product of contemporary biodiversity loss. Chapters 4 and 5 will further explore these issues. Meanwhile, it is unusual to find a population of infants with low levels of this genus, and these findings illustrate a need to expand current standards by which gut profiles are classified as 'healthy' or 'unhealthy', based on assumptions about the taxa that may be context-dependent. If found to be a product of contemporary lifestyles, this research may indicate the need for urgent policy and probiotic intervention.

Feeding practices influenced both the α-diversity and the relative abundance of microbial composition in this population more generally. In the first months of life, breast-fed infants had significantly lower levels of α-diversity when compared to formula- and mixed-fed infants, as well as different compositional profiles. Breast-fed infants harbored substantially higher levels of

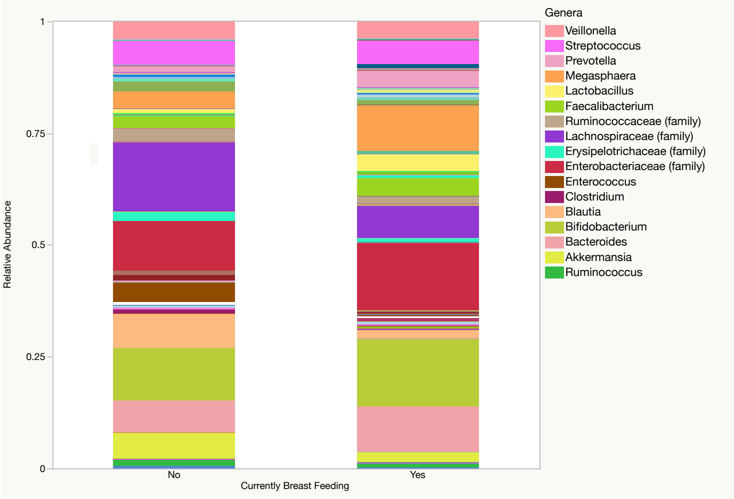


Figure 19: 100% stacked bar graph of bacterial taxonomic composition among currently breastfeeding infants, and fully weaned ones, age 8-12 mo.

Fully weaned infants harbor more Enterococcus, Lachnospiraceae, Blautia, Akkermansia and Ruminococcus, while breastfeeding infants harbored more Prevotella, Megasphaera, Lactobacillus, Enterobacteriaceae, Bifidobacterium, and Bacteroides.

Bifidobacterium, *Lactobacillus*, and *Veillonella* than formula-fed infants. *Akkermansia* appeared earlier in BF infants, and high levels of pro-inflammatory *Blautia* were only found in FF infants. The cessation of breast-feeding had profound effects on the microbiota in 12-month-old infants and shifted the microbial ecology toward an adult-like composition. Results suggest that the cessation of breast-feeding may be a major transition in the development of the adult microbiome, even more so than the introduction of solid foods (see Figure 19).

The cessation of breast-feeding had profound effects on the microbiota in 8-12-month-old infants and shifted the microbial ecology toward an adult-like composition enriched in Lachnospiraceae*, Enterococcus, Akkermansia, Veillonella,* and *Blautia* (Figure 19). In contrast, the gut microbiome of infants breast-fed at 12 months was still dominated by *Bifidobacterium*, Enterobacteriaceae*, Bacteroides, Lactobacillus*, and *Megasphaera*, many of which are associated with breast milk microbiota (Jost et al. 2014). Similar to other studies, the “microbiota age” of 12 month olds who continued to be nursed appeared younger than that of infants who were no longer breast-fed (Figure 19).

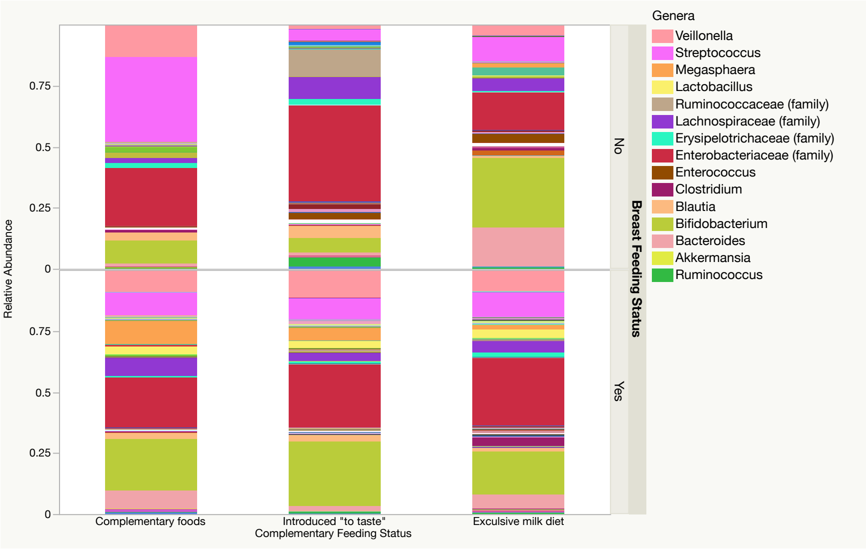


Figure 20: 100% stacked bar graph of bacterial taxonomic composition among infants age 0-3 mo.

Infants who are breastfed (0 mo.-6 mo.) show few alterations in their microbiota when foods are introduced (while receiving umbrella protection from breast milk). Infants who are no longer receiving breast milk show increased variation in microbial community composition as complementary foods are introduced. Infants who are not breastfed and are fed complementary foods in these early months show high levels of pathogenic microbes including Streptococcus and almost no Lactobacillus.

Moreover, the cessation of breast-feeding seemed to have a greater effect on community composition than the introduction of complementary foods in breast-fed, but not formula-fed infants at 0-6 months (see Figure 20). Thus breast milk may provide a primary source of microbial stability during the introduction of complementary foods and other horizontal exposures occurring in the second half of the first year. These results underscore the important role of breast-feeding in the shaping and succession of gut microbial communities during the first year of life, and are in alignment with other studies exploring the microbial correlates of the weaning transition in US and European infants (Bäckhed et al. 2015; Thompson et al. 2015).

Importantly, C-sections represent a barrier to other forms of vertical transmission by blocking other important postnatal practices, including maternal-infant skin-to-skin contact and the early initiation of breast-feeding, factors that have been shown to influence the duration and exclusivity of breastfeeding (Vaidya et al. 2005; Moore and Anderson 2007), another key variable that this chapter has shown to affect infant microbial assembly. Colostrum, the first milk produced by the mammary gland, and then breast milk are a primary and constant source of commensal and mutualistic bacteria designed to coat the infant gut in the first months and years of life (Rocío Martín et al. 2003; Heikkilä and Saris 2003; Jiménez et al. 2008; Rocío Martín et al. 2005). An infant consuming approximately 800 mL/day of milk would ingest between 1 × 105 and 1 × 107 bacteria daily (Heikkilä and Saris 2003), explaining why a breastfed infant's microbiota closely resembles their mother's breast milk microbiome (Favier et al. 2002). Moreover, human milk oligosaccharides (HMOs), or complex sugars, are a major food source for key infant intestinal microbiota (Zivkovic et al. 2011). Yet C-sections infants are less likely to be breastfed than vaginally birthed infants (Hobbs et al. 2016). Finally, C-sections have been shown to directly affect breastmilk microbiome diversity, likely a factor in lower diversity levels among infants who were breast-fed and yet cesarean delivered (Cabrera-Rubio et al. 2012).

These insights combined with the observation that many bacteria known to provide an early capacity for milk metabolism are often exclusive genera to vaginally birthed infants (Fredrik Bäckhed et al. 2015) indicates that the microbes acquired during vaginal birth may help prime the microbiome for a breast-milk diet. In other words, breastfeeding may enhance and enrich vaginally acquired lactic acid-producing bacteria in the baby's intestine (Coppa et al. 2006), although the source of the predominant lactobacilli in the baby's intestine is difficult to define (Martín et al. 2007). From an evolutionary perspective, these two factors may work synergistically to support a sucessional development during infancy. This chapter revealed that when these factors were sub-grouped together, such as vaginally delivered, breast-fed infants (VDBF) and cesarean delivered, formula fed infants (CDFF), effects were enhanced. For example, CDFF infants were found to have substantially higher levels of pro-inflammatory bacteria such as *Streptococcus* (22.61% compared to 6.12% in their VDBF counterparts at 4-7 months) and *Blautia* (6.83% compared to 0.79% in VDBF infants at 8-11 months). The CDFF group also experienced a bloom of Lachnospiraceae (23% in CDFF group compared to 3% in the VDBF group), which has been linked to obesity in childhood and adulthood (Cho et al. 2012). Conversely VDBF infants have higher levels of anti-inflammatory bacteria such as *Akkermansia*, *Bifidobacterium, Faecalibacterium, Lactobacillus* and *Bacter*oides all of which are expected in infants of these age groups, and yet were delayed in CDFF infants.

The mechanisms mediating these multi-factorial, synergistic relationships are unclear, and likely vary among bacteria and infants. For example, even though *Akkermansia* is considered a late colonizer, early care practices seemed to affect whether infants harbored this bacterium and in what quantities. CDFF infants never acquired levels of *Akkermansia* above .33% in their first *two years*. Nearly all infants who acquired high levels of *Bacteroides* were VDBF, although neither birth mode nor early feeding were significant independent factors. Infants of the subgroup VDBF had high levels of *Veillonella* in the early months, tapering off over the course of the first year, whereas CDFF infants experienced a delayed colonization until after the first year of life. Conversely, CDBF infants had the highest levels of *Clostridia* at 0-3 months (11% compared to 2.8% in VDBF infants), although unfortunately CDFF did not have enough of a sample size to analyze. Combined, these findings suggest that birth mode and early feeding together may help seed and nurture a successional development of the gut microbiome.

Another key example of this microbial synergism is represented by the colonization patterns of *Lactobacillus*. Birth mode significantly influenced the colonization of this bacterium: without controlling for age, vaginally delivered infants harbor significantly higher levels of Lactobacillus than cesarean delivered infants (F=5.4, P=.02). Early feeding was also a significant factor: Infants who were breastfed and those who were mixed-fed had significantly higher relative abundance of *Lactobacillus* than infants who were formula fed (P=.01, P=.04 respectively). Interestingly, during the first 3 months of life, VDBF infants had the highest averages of this bacterium (2.62%), whereas CDBF had only 0.71%, and VDFF only 0.05%, suggesting that early bacteria seeded through vaginal delivery may be enhanced by breast-feeding. Mechanistically this may be an inter-microbial synergism. Bifidobacterium as a genus is known to create an acidic barrier against pathogenic bacteria, which also creates a favorable environment for Lactobacillus. Thus, *Lactobacillus* and other vaginally associated bacteria that were seeded at birth may flourish only in the presence of other bacteria. It is also possible that mothers in this study population harbor fewer *Lactobacillus* in their vaginal canal, making birth-mode a less significant factor the colonization of this bacterium, as well as a less dominant bacterium in general when compared to other populations (Dominguez-Bello et al. 2010). These hypotheses are in part validated by assessing infants who have higher than the predicted range of *Lactobacillus*: 9/11 of the infants were VD, 7/11 were BF, and importantly none were FF. All outlier infants were generally healthy, and none showed adverse effects associated with high levels of the bacterium. Chapters 4 and 5 further explore the consequences of not having this bacterium present in the early gut microbiome of infants in this population.

The abundance of beneficial microbes supported by breastfeeding combined with vaginal deliveries are thought to provide protection from disorders such as allergies, neonatal diarrhea (Ruiz-Palacios et al. 1990), necrotizing enterocolitis (McGuire and Anthony 2003), obesity (Owen et al. 2005; Cho et al. 2012) and type 2 diabetes (Owen et al. 2006). These studies and current results can only hint at the life-long effects that breast-feeding and birth mode may have on the developing microbiome, with possible consequences for metabolic and immune health that are gradually being elucidated. At the same time, these early factors do not explain as much of the variation as in other populations.

A primary limitation of this study was that small community population size meant that infant ages at baseline ranged from 0-8 mo. These age differences complicated longitudinal analyses, whereas a typical cohort study would have more precise longitudinal analyses of changes based on specific ages. Also, limited sample size made certain statistical analyses impossible when dividing infants into subgroups. At the same time, this study followed nearly 100% of the infants under one year of age in this community, which allowed for a comprehensive exploration of the range of issues for this community of infants at all sampled ages from a cross-sectional and in some instances, longitudinal perspective.

Another limitation was the use of fecal samples to represent the gut microbiome, which may not accurately represent the actual colonization patterns of the large intestine (Zoetendal et al. 2006). As most other studies that focus on gut microbiome, the current study utilized infant fecal matter to represent the microbial composition of the large intestine. Unfortunately, as with any DNA-based, culture-independent study that does not discriminate between live and dead bacteria, the number and identity of bacteria detected in this studies may not represent actual bacterial counts, and should be interpreted with some caution (Zoetendal et al. 2006).

Limitations aside, these results contribute to a rapidly growing field that probes the internal world of the human gut microbiome, as it relates to a broader human ecology. This study illustrates the importance of understanding how contemporary birthing and feeding practices affect the early development of infant microbiomes, not only in wealthy and western countries, but also in resource-poor, developing countries. If it is true that the infant microbiome develops within its socio-ecological context, and that a developmentally perturbed microbiome causes an array of downstream issues with autoimmunity and metabolic disorders, it is of critical importance that researchers explore these early processes within each developmental “niche”. These initiatives will implore global health campaigns to shift focus from infant survival alone, to entire life-course well-being, by protecting early developmental windows from modern obstructions. “Chronic” diseases are not chronic in an impoverished context, but rather they are debilitating and frequently deadly. Excitingly, microbiomics research holds the potential for developing effective, safe and affordable interventions for infants and adults. Even now, probiotic supplements have been shown to decrease colic symptoms (Indrio et al. 2014; Savino et al. 2007; Weerth et al. 2013), to prevent allergies (Koga et al. 2016; Wang et al. 2008), and to treat acute bacterial infections (Henker et al. 2008) in infants. Finally, as this study and others suggest, the power of context and the extraordinary adaptive capacity of human and microbial biology should not be underestimated. To understand what is a “healthy,” well adapted microbiome for a given population, researchers must explore interactions beyond the proximal microbial dynamics, and even beyond the main factors explored in this chapter. This chapter clearly illustrated both the importance of early vertical transmission factors, and also highlighted the enormous amount of variation that was left unexplained. The following chapters of this dissertation explore the social, political and ecological factors that contribute to microbial variation among infants of this small, Salvadoran village.

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CHAPTER 3: A DEVELOPMENTAL ECOLOGY OF THE INFANT GUT MICROBIOME

Introduction

Microbiomics research has intensively investigated the affect of early care practices such as birth mode and early feeding on the development of the intestinal microbiome during infancy (Dominguez-Bello et al. 2010; Bokulich et al. 2016; Bergström et al. 2014). While researchers emphasize the importance of the environment in driving microbial diversity, scant research has thus far investigated how variations in diet, geography, and local ecology may influence the developing gut microbiome (De Filippo et al. 2010; Blaser et al. 2012; Yatsunenko et al. 2012; Vatanen et al. 2016). The current study aims to address that gap, using a bio-cultural, mixed-method and multi-disciplinary approach to explore broader social, political economic and ecological variables currently missing from microbiomics research. While chapter 2 examined the important effects of birth mode and early feeding practices on the early development of the microbiome, this chapter ventures beyond current understandings to investigate how these macro factors shape infant exposures, microbial development and ultimately health.

To explore the complex, multilevel and multifactorial influences on early infant microbial development, this chapter draws from a rich orientation within biological anthropology called developmental ecology. This perspective offers an integrative framework for understanding the roles of individuals, households, communities, populations and evolutionary forces in developmental processes (Worthman 2010). The framework enables researchers to draw concrete connections between developmental processes and eco-social conditions by operationalizing comparative research within and between populations (Ibid). A key strength of this orientation is the emphasis on theoretical specificity and the generation of testable hypotheses (Worthman 2010; Whiting et al. 1975). This chapter draws from this established body of work to explore less charted territories of microbiomics research.

This chapter identifies four key levels of analyses to explore these relationships: A macro level, the household unit, proximal mediators, and the micro level. At the macro, gendered labor markets, the demographic transition and socioeconomic status are factors that relate to broader national and global economies, and yet have direct influence on the daily lived experience of families in Joya de Cerén. These three macro processes are each examined in relation to their affect on the other three levels of analyses and ultimately the infant gut microbiome. The household level describes different characteristics of household composition and dynamics that shape early infant exposures. Household level factors make up a Household Microbial Ecology Index (HMEI), which is a composite index of microbial exposures through housekeeping, childcare practices, household construction and maintenance, and sanitation systems. At the mediator level are two mechanisms by which microbes are transmitted to the developing microbiome: the early feeding mediator represents feeding ecologies (i.e. caretaker beliefs and practices regarding infant feeding); microbial ecology describes the material environment(s) where microbes are transmitted to the infant body through contact. The latter is almost entirely shaped by HMEI factors. Finally, at the micro level is the infant gut microbiome. This chapter necessarily snakes through a complex web of interconnected factors from all levels. To clarify these complex relationships, Figure 21 represents the main model of this chapter. Each section of this paper will explore a specific subset of the broader model.

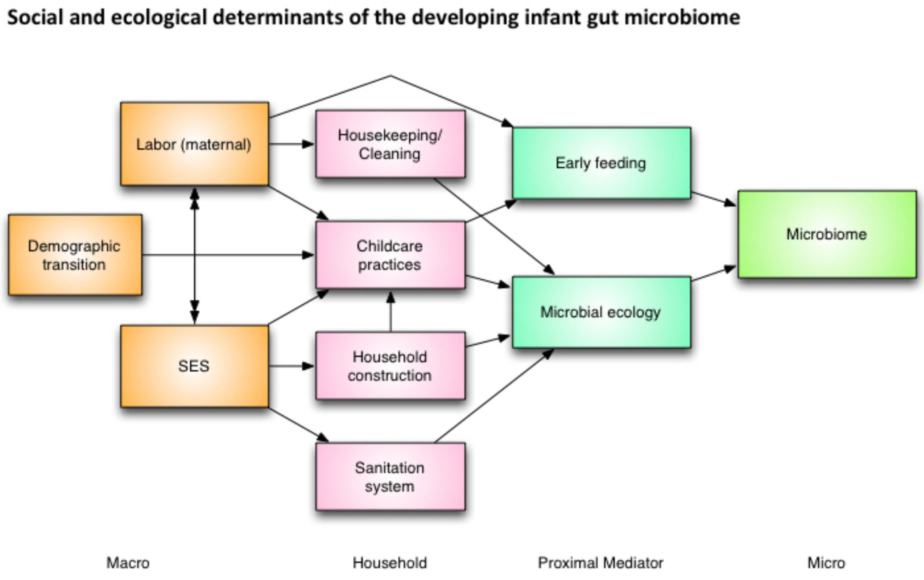

Figure 21: Model of the social, political and ecological determinants of the developing infant gut microbiome.

This flow chart represents the pathways of microbial colonization for infants in this semi-rural Salvadoran population, from macro to micro levels. At the macro level are gendered labor markets that hire only women between the ages of 18-30, a demographic transition that has transformed social and childcare networks, and socioeconomic status (SES) that affects many aspects of the household functioning. At the level of the household, housekeeping and cleaning practices, childcare networks and practices, material aspects of household construction, and sanitation systems greatly affect variables at the proximal mediator level. These mediators include the early feeding ecology and microbial ecology. Early feeding ecologies (caretaker beliefs and practices regarding early feeding) together with microbial ecology (material environments where microbes contact the infant body) represent the main pathways recognized in this chapter by which infants acquire their microbiota in the first year of life.

The principal hypothesis of this chapter proposes that *differences in household ecologies (shaped by broader socio-political and economic factors) will produce differential microbial exposures and therefore variation in microbiome development*. To explore the theoretical basis of this assertion, this chapter is organized into two main sections: section I contextualizes and defines the complex relationships among the different elements of the main model (see Figure 21). This section details the history of the planned community, and explicates recent social and economic transformations that have changed the daily lived experience and behaviors of inhabitants and study participants. Here, the household becomes a central unit of investigation, a space within which broader powers and processes exert their control, and where beliefs and practices operate to influence the first microbial exposures that infants encounter. This section elaborates the construction, maintenance and infrastructure of households, how these constructed ecologies facilitate the daily flow of people, goods and microbes. Shifting labor patterns, gender roles, demographic changes are all examined as they relate to infant care practices. Finally, two key multi-factorial indexes are described, specifically, socioeconomic standing (SES) and the Household Microbial Ecology Index (HMEI). Examples of households that typify each level are elaborated. Finally, an early feeding ecology of this population is described. Maternal and primary caretaker beliefs are discussed and compared to actual practices.

Section II outlines hypotheses and research questions generated from section I (in relation to infant microbial exposures and bacterial development), and tests them. Methods are specified in this section, and results are detailed.

Lastly, patterns among these disparate elements of the overall model are reflected upon, helping to illuminate key patterns and pathways by which microbial exposures and infant microbiota are influenced by broader political ecologies.

Section I: Study Population and microbial ecology contextualized

Joya de Cerén is a semi-rural community of approximately 6313 inhabitants, located 30 miles from the capital city of San Salvador, El Salvador. Nestled in a lowland valley, this region has a tropical climate with pronounced wet and dry seasons. Over 85% of the population identify as “mestizo” or mixed Indigenous Native American and European Spanish origin, another 12% identify as mostly European descent, while the remaining 1% identify as purely Indigenous descent (*Pipil* or *Lenca*). This population has largely transitioned from subsistence-based agriculture to a struggling market economy. Large family sizes of prior generations have meant high-density living. Households are poor, with an average of 6 people living in each 1-2 room dwelling. Unemployment rates are high, and commonly the income of 1-2 adults must financially support the entire household.

Household construction; then and now

The community was initially founded in the early 1950s as an agricultural reform project. Uniquely, planners from the Instituto Salvadoreño de Transformacion Agraria (ISTA), not only thought about agricultural and economic systems, but also sought to create a social organization that would help develop and maintain a sense of community. The project founders considered the most important unit of this community to be the family, and so they set out to design household constructions to be supportive of this primary unit (Lara 1998).

Figure 23: Layers of new construction materials for the poorest families in Joya de Cerén.

The original houses of the 1954 construction were on lots that were approximately 50 x 100 meters (164 x 328 feet) and built of cinderblock. Many changes have been made to the initial developments over the following seventy years; sections of lots have been sold over time, and/or layers of new construction have been built to accommodate growing families of later generations (Figure 23). Currently, 76% of study participants live on land that is owned by their own or their spouse's family, while only 24% of participants pay rent. Very few study participants personally own the land they live on. One father had previously worked as a mercenary, and another migrated to

Figure 22: "Pilas," or cement sinks are used for washing dishes, clothes and babies.

the U.S. for work, and both returned with accumulated funds to purchase land for their families, although this is unusual in the area.

Commonly new construction is built around a communal space. The households function with differing degrees of autonomy, and often maintain a certain degree of mutual social and financial support. Some of the more traditional (often lower socioeconomic status) households share a kitchen that is external to all structures, located a few meters from the dwellings. This plan supports the sharing of space, and helps to prevent smoke from the wood fire from entering the houses, while simultaneously decreasing the likelihood of homes catching fire. Of central importance to the kitchen, is a large cement sink called *pila* (see Figure 22), where dishes, clothes and babies are washed, and where members of the family bathe. The most modern houses of the highest SES families now have showers with running water, although this remains a rare luxury. Tucked in the furthermost corner of each property is an outhouse, and sometimes in the case of wealthier families, a flush toilet. Almost all households have grey water systems that lead out to ditches that run along the streets, ultimately reaching the río Sucio ("dirty" river). Many of the middle and upper socioeconomic households are connected to Joya's central water system. The water is treated in Joya at a local facility and distributed through pipes. Some of the poorest households do not have running water, but instead have plastic drums that are filled at a neighbor's house and used for water needs. If there is unbuilt land on the properties, they often contain a plot of diverse fruit trees and other vegetation. If the family maintains a *milpa*[3], it is usually a 30-45 minute walk from their residence. The previously mentioned father who migrated to the U.S. for work purchased a small *milpa* at the entrance of town, although this is now the exception.

Household construction and child care networks

As previously noted, a great majority of couples move in with family members. Most of these couples live with the husband's family, which constitutes the extended family dynamic and main basis of childcare networks for many of the households of Joya de Cerén. A young couple usually will construct an independent dwelling on the property of the husband's family. Although this unit is physically separate, it shares common spaces to facilitate the exchange of goods and services with the other units and family members. This new family unit will often maintain some degree of economic independence, when possible. Few nuclear families live independently however, and older relatives are often moved into the household when necessary.

Childcare networks are made of these webs of relationships, which often include those living on the same or adjacent properties. Notably, because couples often move into the husband's family property, it is common that his family

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[3] Milpas in this area are small subsistence farming systems where corn, beans and squash are grown together for household consumption and in some cases for sale.

members (often his mother and sisters) participate to some degree in childcare. As relationships are not immutable, care networks are also dynamic and fluid based on emotional ties, work, family dynamics and other life events experienced throughout the web. Childcare networks and practices are subject to broader social, demographic and economic changes. The following explores how these broader processes, such as gendered labor patterns, SES levels, and paternal care patterns, influence both the material spaces that children inhabit and the care programs that affect infant microbial ecology on a daily basis.

The demographic transition and thinning child care networks

Sibling and cousin relationships, particularly on the maternal side, have traditionally made up an important part of the extended infant care network. Recent generations, however, have experienced dramatic decreases in family size, as Salvadoran women have fewer children and utilize more family planning (including injectable contraceptives). A 2008 family planning survey by the Salvadoran Ministry of Health found that fertility dropped 60 percent over the last 30 years, from 6.3 lifetime births per woman in the mid-1970s to 2.5 in the 2003-2008 period (Ministerio de Salud El Salvador 2009). While women in rural areas average 1 child more than those in urban areas (3.0 compared with 2.1 on average) the average number of children per participating mother in my study sample was exactly 2 children (standard deviation 1.2). These trends towards low fertility in Joya de Cerén can be in part attributed to high rates of contraceptive use and availability. There remains, however, a fertility gap between the wealthiest and the poorest women of Joya de Cerén: 1.6 and 5.0 children, respectively.

The decrease in family size also has lead to a thinning of infant care networks for mothers. Older women (often grandmothers) explained how their sisters and cousins made up robust childcare networks that helped raise their children. In this increasingly monetized economy, family members can no longer afford to take care of other people's children, but must work to support their own families. The web of care increasingly has shrunk due to changes in market integration, the increased number of women in the workforce, and decreasing family size. Mothers of this study reported an average of 2.5 other family members or friends who helped care for their children, ranging from 0 to 7. Only one study infant of a working mother spent her days with a paid caretaker.

Gendered labor patterns, the household and childcare networks

With the establishment of international free trade policies, such as the Central America Free Trade Agreement (CAFTA) and GATT, transnational corporations have expanded their factories ("maquiladoras") in developing countries in search of cheap labor. These corporations prefer female labor over male labor because women are considered "docile," cheap workers. Because women are not regarded as primary economic provider (although increasingly they are), and because factory work generally is considered to be an extension of female household roles, women often paid less for long hours. Hence, El

Salvador, like other developing countries, has experienced a recent surge in employment opportunities for women in the formal market sector (Freeman 2000; Moghadam 2000; Boserup and Kanji 2007).

Such high labor demand with employment opportunities for women has created many and substantial changes within the social structure of Joya de Cerén. On one hand, the demand for female employment provide women with increased opportunities and a sense of independence. Scholars warn however, that the glass ceiling is low due to the "feminization of poverty." Low wages earned by female workers provides only for survival, particularly when the woman is the sole provider for her family (as is true for many Joya de Cerén residents). These issues are further exacerbated by the fact that factories only hire women of child-bearing years (18-30yr). Changing labor markets affect not only gender relations, but also childcare practices within the community and among different households.

In both developing and developed countries, maternal labor patterns have been shown to influence rates of breastfeeding. Although the quantity and quality of breast milk are not undermined by maternal work or activity (Lovelady et al. 1990; Scott and Binns 1999), it is often added time constraints and labor conditions that working mothers cite as the reasons for early supplementary feeding and/or weaning infants (Gielen et al. 1991). In many cases, a woman's ability to breastfeed is greatly inhibited when she returns to work if she does not have adequate breastfeeding or pumping breaks, if quality infant care near her workplace is inaccessible or unaffordable, and if not facilities are available for milk pumping or storing (Gatrell 2007).

Increased paternal participation

Figure 24: Paternal participation in caretaking.

On his day off from factory work, this young father gives his son a bath.

Ethnographic investigation and participant observation revealed a general increase in paternal participation in childcare. Without data from earlier time periods, it is difficult to indicate how much paternal participation has increased, however memory and anecdotal evidence suggest this is a relatively recent trend among fathers. During home visits fathers were often observed holding, playing with, or caring for their infant. Fathers expressed varying degrees of embarrassment when observed, at some points handing off the child to the mother or other caretakers. Other fathers were quite adamant about care practices and proudly cared for their infants. When inquiring about father participation, mothers often discussed paternal work patterns as a main reason that their partner could or could not participate in child care. It appeared that changes in the labor market may influence this changing childcare dynamic.

Socioeconomic status

Socioeconomic status (SES) is commonly conceptualized as the social standing or class of an individual or group. Differences in SES often are associated with inequities related to privilege, power and control, often resulting in differential access to resources. The relationship between SES and health is strong and well established (see Anderson and Armstead 1995; Marmot et al. 1995; Marmot et al. 1984; Townsend et al. 1988), although few researchers have investigated the effects of SES on infant microbial ecology and development. In this section I explore the hypothesis that SES will influence multiple socio-cultural and ecological variables that in turn influence infant care and microbial exposure, and thus infant microbial development (see Figure 26).

SES was measured from both subjective and objective measures. The subjective measure was adapted from the MacArthur Scale of Subjective Social Status. This measure was developed to capture "a person's belief about his location in a status order" (Davis 1956) or socioeconomic structure. During the interview, an image was presented of a "social ladder" and asked participants to place an "X" on the rung on which they felt they stood. First, participants

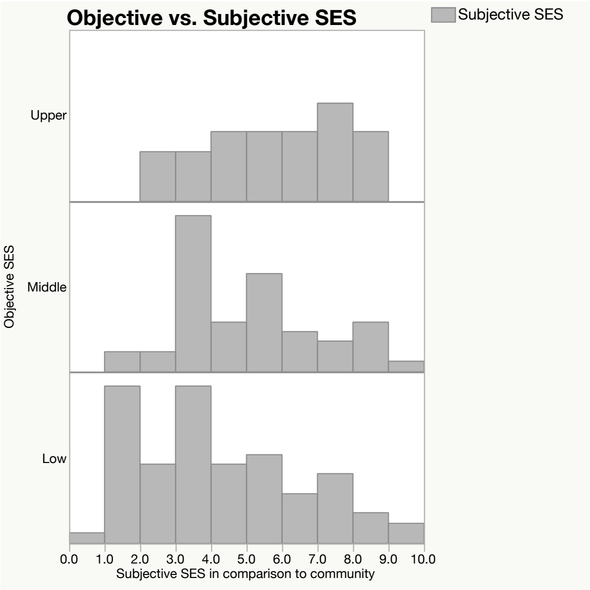


Figure 25: Objective vs. Subjective SES measures.

Some participants who rated high on the objective SES measure rated themselves lower on the subjective measure, and the opposite was true of lower objective SES participants.

were asked about their standing in relation to their community (as they defined community), followed by their standing on the ladder in relation to their country. Scholars have suggested that subjective measures of SES may be of particular interest in poorer communities in which individuals may not be high on the SES ladder in terms of income, occupation, or education, but may have high social standing within their social groups such as a religious or local community (Adler et al. 2000). Insofar social standing has beneficial effects on biological processes related to health, standing on the community ladder may be as important as higher objective SES.

The objective measure of SES was a composite of multiple social and economic variables including mother's education, maternal employment status, the number of economically active individuals and the number of dependents within the household, the household status (owned, rented, living free of rent, etc.), monthly household income, and family remittance. Objective SES is typically broken into three categories (high, middle, and low SES) to describe the three levels an individual or family may fall into. Stratification criteria can be based on observed distinctions among households.

Similar to findings in Adler et al. (2000), there was a significant but imperfect relationship between objective and subjective indicators of SES, and the relationship leaves nearly 80% of variance unexplained (R2 =.22, p< .0001). This result is consistent with the literature, which shows that ladder rankings most often reflect but are not redundant with the objective indicators. Literature has suggested that different components of SES are measured and valued differently across individuals (Adler et al. 2000). In this Salvadoran population, it is likely that other socio-political factors influence the variance. For example, Figure 25 indicates that participants who were ranked high on the objective SES scale tended to rate themselves lower on the subjective scale, and visa-versa with lower objective SES members. High SES residents suggesting lower subjective measures can be partly explained by the socio-political climate of the community. Joya, like most other places in El Salvador is controlled by gangs who survive through extortion of villagers. Individuals and households with more income will under-estimate their wealth, while pride often encourages the poorest families to declare higher subjective SES. For these reasons, objective SES was exclusively used for assessing the validity of **H3**.

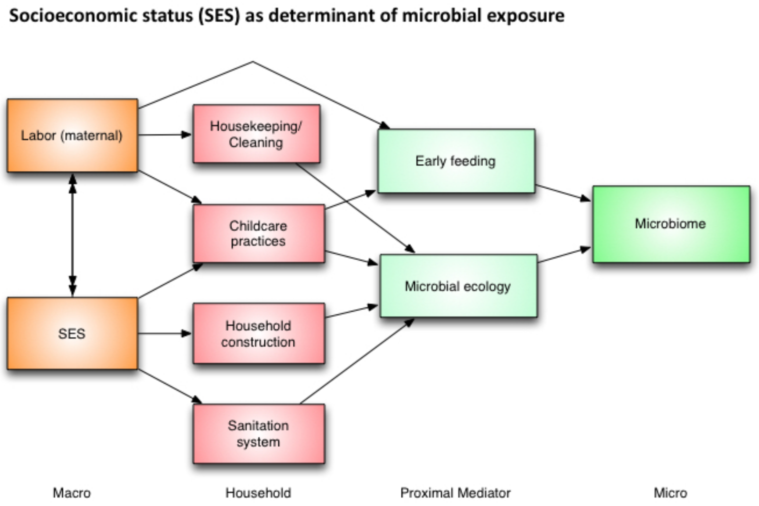


Figure 26: Socioeconomic status as a determinant of microbial colonization.

SES can affect many aspects of infant microbial ecology. In this context, it dictates what jobs a mother will have access to, her ability to provide for and maintain the household, whether she is able to breastfeed her child.

The next section profiles the households from each of the three socioeconomic levels in this study population, followed by analyses of **H3**.

Low SES households (39.4% of sample)

The lowest SES households are often called “los ranchos” and houses are constructed of layers of cardboard, tin, cloth, plastic and other materials strapped together with hard-packed dirt floors (see Figure 28). These houses are made up of one larger room informally divided into the living room/bedroom and the kitchen or food preparation area. This room does not usually have a window, and without electricity family members spend most of the day outside in a covered porch adjacent to the house. The poorer households have wood burning stoves. Holes in the old tin roofs allow sunlight to filter into the home, although they also do not obstruct rainwater that drips inside. Hammocks are often strung across living rooms and porches and are used to rock babies to sleep, or for family members to lounge in the absence of sofas and other furniture. These houses generally have an outdoor wood burning stove, a pit latrine, and no running water. In houses that do not have running water, plastic drums are kept outside. Upkeep of these poorer households ranges from meticulously swept to littered with piles of discarded objects. Households with higher density of inhabitants tend to be less well kept.

Figure 27: Photo of a well-maintained, low-SES household.

This photo is of one study participant from the low SES level. She is a single mother with five children. She lives on her father's property in a well-constructed home that is well cared for. (Photo credits to Fran Mandel Sheets, 2013).

Households with slightly more means, while still in this SES range, are often of mixed construction materials; many are constructed in part of adobe (sun-dried, not fired), bahareque (wattle-and-daub), and increasingly of fired red brick (see Figure 28). These houses often have an open window or two allowing natural light, some air, and also creatures (insects, rodents, pets) into the household. The windows are often open, but may be covered with an iron grill anchored to the outside wall (see Figure 27). Such houses are often one room, but can be two-room constructions. Most commonly these houses do not have gas, electricity, running water, or flush toilets. The floors are often compacted dirt. Some of these houses are brightly painted to reflect care.

Often study participants from the lower SES level have attained lower levels of education, and have higher numbers of dependent individuals in the household. These households are often single mothers or those with husbands who are underemployed. These mothers often participate in an infrequent sale of timely products such as seasonally dependent fried yucca, or harvested herbs. Economic insecurity is a persistent source of stress for these mothers.

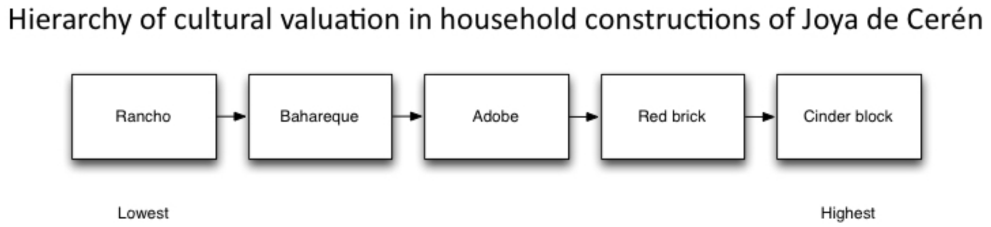


Figure 28: Hierarchy of cultural valuation of household construction.

Flow chart illustrates the cultural valuation of different construction materials of households in Joya de Cerén (modified from Lara 1998).

Middle SES household (26.8% of sample)

Many of the middle and upper SES houses in Joya are now constructed with cinderblock or red brick, tin roofs, and concrete or tile floors (see Figure 28). The more modern constructions of brick or cinderblock are the most desirable in town because they are the most durable and resistant to the tropical climate and earthquake. These contemporary constructions also signal social and economic power amongst community members. Thus, those that have economic capacity substitute their adobe or *bahareque* houses for mixed brick or cinderblock, indicating that they have attained a certain socioeconomic status. That way the construction system signals economic and social power to the rest of the canton.

Houses built in the middle SES range tend to be well constructed with red brick or cinderblock, tin roofs and a cement or tile floor. A middle SES house typically has at least two rooms, one living room/kitchen and one bedroom. The interior of these homes in Joya de Cerén is generally quite simple. In all but the poorest households there is a plastic or wooden table that is used as a surface to cook, eat and work at (kids usually do their homework here), with plastic stackable chairs of varying colors and conditions. These houses have windows, electricity and tend to have running water. Kitchens are often well outfitted with gas stoves, blenders and possibly other kitchen appliances. A few of these homes now have televisions and/or stereo systems. Pit latrines are still the norm in these families. Laundry hangs outside; the upkeep of the grounds range, but are often neatly kept. There might be some landscaping, or a sort of yard and garden.

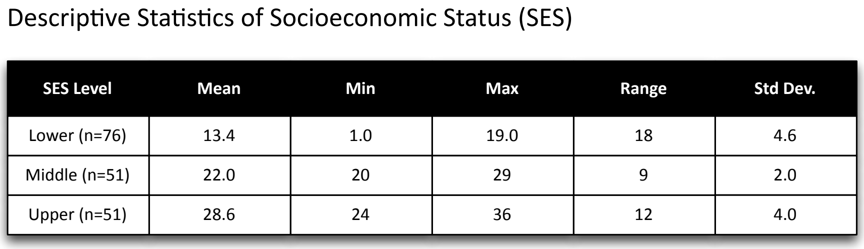
These households often have two-parent incomes, or a single mother who is formally and somewhat stably employed. Some mothers in this level are single, and work at the family store, or have an informal business themselves. These households range in the number and ages of dependents.

High SES households (29.6% of sample)

Upper SES households tend to be of more complex architectural design with multiple rooms that can accommodate an established home business (most often a food store, but in one case a hair salon). These houses have full living rooms with televisions, stereo systems, sofas and entertainment centers. There are usually two or more two bedrooms and a basic kitchen with a gas stove, refrigerator, running water, and various electrical appliances. Cloth curtains in doorways still often separate rooms. Bedrooms are furnished with multiple beds and armoires for clothing. Patios are often well kept and planted. Bathrooms are a mixture of pit latrines and flush toilets.

Upper-middle SES households tend to have elaborate gates, including cement block or brick walls around the perimeter of their homes. Such well-defended boundaries contrast to lower and many middle SES homes that have fewer barriers surrounding the property. Lower and middle SES homes sometimes have a wooden pole fence with barbed wire to indicate property lines. This makes the home more visible and vulnerable to intrusion by a trespasser. The highest SES households have 6-8 foot cement walls (some of which have razor wire or other security precautions) surrounding the perimeter of the properties. These walls obscure the property from view and are intended to increase security and prevent intrusion.

Affluent households have extra money to spend in other areas. For example, these families often purchase different kinds of processed and imported foods that represent higher status. These families can afford private medical clinic visits, which above all else are time-saving. The high SES group in this study does not represent the highest SES levels of the general Joya de Cerén population. The wealthiest residents of Joya tend to have family or have sojourned abroad themselves to work for a period of years. They have returned with a wealth that is not easily attainable within the rigid socioeconomic structure of El Salvador.



Descriptive Statistics of Socioeconomic Status (SES)

SES Level	Mean	Min	Max	Range	Std Dev.
Lower (n=76)	13.4	1.0	19.0	18	4.6
Middle (n=51)	22.0	20	29	9	2.0
Upper (n=51)	28.6	24	36	12	4.0

Table 12: Summary statistics of socioeconomic standing.

SES ranged from 0-36 on the scale and included metrics such as education, maternal work status, household status, monthly household income, and family remittance.

Mothers in this group likely have attained high levels of education, and are often currently formally employed or were in the recent past. Single mothers are rare in this population, and often these are two-income households. Fathers are often employed in the formal sector. These couples often rent a small house for their nuclear family. Not living on family-owned property means that these households are less likely to have older or younger relatives living with them, making household density low. Some of these families receive remittance support from relatives abroad.

Microbial Ecology and the HMEI Index

Microbiomics researchers have suspected that material environments shape the development of the microbiome. In order to probe this notion, investigators have drawn cross-cultural comparisons, showing that people living in different parts of the world have diverse microbiomes (Yatsunenko et al. 2012). Yet comparisons such as these draw broad strokes and deeper understandings of the local ecologies that shape the inter- and intra-population variations have yet to be established. The study design of these projects has not supported this clarification, as they are commonly cross-sectional and quantitatively focused. In order to understand how local ecologies contribute to the development of infant microbial gut communities, ethnographic research of daily lived conditions is required. This study is unique in that I explore the differences in microbial exposures within a single community, which permits controlling for many factors such as climate, geographic location, and other cultural factors that likely cloud cross-cultural investigation. Additionally, I was fortunate to visit families in their homes for interviews, participant observation and casual visits over the two years that I conducted research in this village. Local collaboration further enhanced my understandings of household dynamics. The central importance of the household unit both to this community and to a child's early

Figure 29: Dirty feet of two brothers from a low HMEI (high exposure) household.

ecology made it an ideal level at which to investigate and explore interactions between infants and their environment.

To explore household ecologies in relation to microbial data, I constructed a tri-level scale called the Household Microbial Ecology Index (HMEI). This composite variable is an ethnographically and materially informed assessment that is a negotiated score based on a range of parameters; including household cleanliness and maintenance, sanitation infrastructure of the household, access to running water, household construction (i.e., dirt, tile or cement floors), grounds care (if there were breeding grounds for insects and rodents), animal husbandry or pets (what kinds of animals were allowed into household spaces if any, and if babies were allowed or taught to interact with them). Qualities of infant care that were assessed included, whether mothers/caretakers allowed infants to crawl or play on the floor (either inside or outside the home), or if infants were consistently held or kept on furniture until they learned to walk. Bathing care and hygiene were also factors considered in the HMEI index. Future research could further probe infant care practices by exploring other qualities of care and contact among caretakers and infants. An individual child will experience many changes in

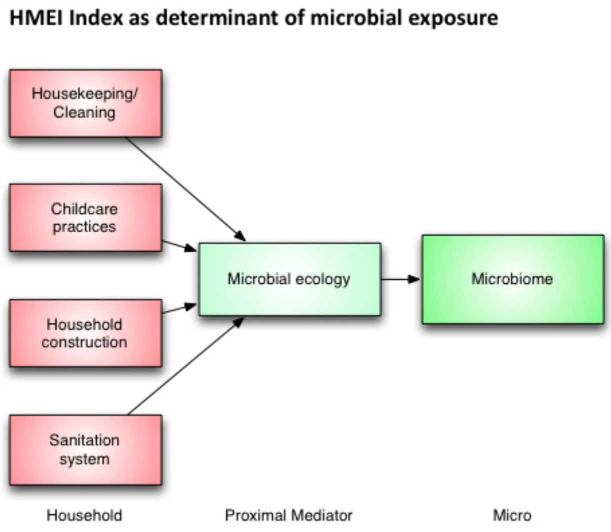


Figure 30: HMEI scale as determinant of microbial exposure and colonization.

This slice of the main model details the embodiment of microbial variation based on the Household Microbial Ecology Index (HMEI).

the type and quality of care over the course of the first years of life. These changes are determined by the child's changing needs but also the life circumstances of the family and caretakers, and finally by the evolving dynamics of care networks. Overall this HMEI model is visualized by Figure 30.

Under the conditions it was not appropriate to ask about each of these factors individually. Instead field notes were combined with expert local knowledge to create this composite index, by stratifying three the levels. Final scores were negotiated or confirmed with two local public health workers who entered these households each month, and were trained to observe many of the aspects that make up the HMEI scale (including searching out and treating water for dengue, observing sanitation systems, consulting with mothers about breastfeeding and care practices, weighing and vaccinating babies, and providing contraceptives, etc.).

HMEI and SES household distribution

HMEI level	SES level
Lower (n=10)	Lower (n=28)
Middle (n=25)	Middle (n=19)
Upper (n=23)	Upper (n=21)

Table 14: Summary statistics on HMEI and SES.

It would logically follow that the HMEI scale would be closely related to an SES index. I tested whether SES and HMEI levels were significantly related, and they were (F=29.7, P=.0001), at all levels. At the same time, the two indexes are not identical and measure different

HMEI and SES

HMEI level	Mean SES	Min SES	Max SES	Range SES	Std Dev. SES
Lower (n=26)	14.5	7	21	14	5.0
Middle (n=63)	21.0	9	35	26	5.9
Upper (n=62)	24.9	12	36	24	6.1

Table 13: HMEI vs. SES.

Middle and upper HMEI levels have similar mean SES, indicating that SES and HMEI measure slightly different variables. SES focuses on material wealth, while HMEI is focused more on upkeep and sanitation levels of households. Interesting that Lower HMEI group only ranges up to a max SES of 21, indicating that this is a poorer group. n=number of total observations.

factors (see Table 13). Only 10 households (26 individual observations) fall into the “low HMEI” category, while over 28 households (76 individual observations) fall into the lower SES category (Table 14). See Table 13 for more details on how these two indexes were related.

Low HMEI Households (17% of sample)

Figure 31: Low HMEI (low sanitation/high exposure) household.

The little boys play naked on the floor next to the counter where cheese is made, kept and sold. Flies, other insects, rodents and pets abound. These kids are bathed every few days.

Homes that are low on the index often have messy, dirty floors with food bits, trash and other items that attract flies, mosquitos and rodents. Infants are often left to roam and play on the floor (see Figure 29 and Figure 31). They are bathed every few days, and when they are it may be amidst trash and food pieces that have accumulated in the pila. These infants often have mothers who are busy working informal market sector jobs, such as making and selling food (tortillas, cheese, or pupusas), or running informal stores that sell miscellaneous items. Parent supervision is often distracted and otherwise occupied with work, other children or other concerns. These children are left to explore their environment more completely, often pick up food and other items from the ground. Older siblings may take on caring for the infant, providing an array of childcare practices and more diverse microbial exposures. The floors of the home tend to be dirt with crumbling adobe or brick walls.

Belongings are strewn around the house and tables are covered with food crumbs, wrappers, trash, and household items. Food storage and preparation is disorganized. Animals are allowed to inhabit the same living spaces. Cats, dogs, chickens and ducks often roam these areas in search of food. Many of these households do not have running water and have outhouses that attract flies.

Middle HMEI Households (43% of sample)

Figure 32: A little girl from a middle HMEI household (middle sanitation/exposure).

She is well groomed and wearing shoes, but she is also permitted to play with animals and interact with her environment.

Households that ranked in the middle of the HMEI index may be anywhere on the spectrum of household construction materials and sanitation infrastructure, but the households are more neatly kept. The households may have lower population density than low HMEI homes. Children are bathed daily, although childcare is far from compulsive and children are left to play and interact with their environment (see Figure 32). These households may have areas of the house or yard that have piles of trash and discarded items where children and animals play. Often these households keep animals and pets that are allowed to roam inside and outside of household spaces.

High HMEI households (38% of sample)

Households that are higher on the HMEI scale tend to have running water, flush toilets and may have simple septic tanks. The most common variable among these households is fastidious house cleaning and infant care. Infants are bathed daily. Floors are swept every morning and sometimes in the afternoon depending on the season (in the dry season the dust from the roads must be swept). Yards are raked or swept daily of falling leaves, ash and debris. Higher HMEI homes tend to have tile or cement floors. Animals are kept out of living areas. These caretakers often do not allow their children to touch the floor and hold the baby or tie them to a chair when they are not being held. Several infants from this HMEI level were never put on the ground and did not learn to crawl (see Figure 33).

Figure 33: Little boy from a high HMEI (high sanitation/low exposure) household.

This photo illustrates the cleanly swept tile floors of a household from the high HMEI group. This child is also bathed daily and has a place to sit on the outdoor patio, at least partially isolated from environmental exposures.

Early Feeding Ecology

Chapter 2 demonstrated the importance of early feeding practices in shaping the composition of the infant microbiome. Breast milk is a primary and constant source of commensal and mutualistic bacteria designed to coat the infant gut (Rocío Martín et al. 2003; Heikkilä and Saris 2003; Jiménez et al. 2008; Rocío Martín et al. 2005). A baby consuming approximately 800 mL/day of milk would ingest between 1 × 105 and 1 × 107 bacteria daily (Heikkilä and Saris 2003), explaining why a breastfed infant's microbiota closely resembles their mother's breast milk microbiome (Favier et al. 2002). Moreover, human milk oligosaccharides (HMOs), or complex sugars, play a key role in driving the community composition and diversity of the infant gut microbiota (Sela and Mills 2010). HMOs are the third-largest solid component of milk and because of their structural complexity they are indigestible to the host, making them a major food source for infant intestinal microbiota (Zivkovic et al. 2011). Formula fed infants in this study showed unstable and delayed colonization of important successional microbes that may have key roles in the development of gut and immune health. (see Chapter 2).

Breastfeeding is both a biological and cultural endeavor. Social and cultural factors can have a profound influence on whether or not a mother will choose to, or be biologically capable of, breastfeeding her child. This section is meant to contextualize the early feeding ecology by exploring beliefs, practices, and structural factors that influence infant feeding in this study population.

Beliefs about appropriate infant feeding

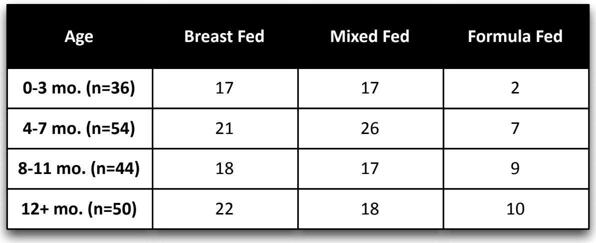
Interviewed mothers unanimously agreed that breastfeeding is beneficial to an infant's health. Nearly all mothers said that it was the best possible food for infants. Reasons for this proposed superiority varied, although the most common answers were that breast milk is healthy for babies, they get sick less often, it makes them stronger and gives them *defensas*, or immune defense. Other mothers said that breast milk offers babies specific nutrients and vitamins that formula does not have. One mother said that breast milk does not harm her child's teeth [in comparison to formulas and added sugar in bottles]. Fully 88% of mothers interviewed said that colostrum was very beneficial for newborns and that it should be administered as soon as possible after birth. A common response was that "it is the best breast milk, with the most nutrients." Most mothers said that formula is not as good as breast milk because it is not "natural." One mother said that it is because there are chemicals in formula.

Mothers who exclusively breast- or mixed- fed their infants often looked perplexed when I inquired about their reasons for breastfeeding. After a pause they would often answer that it was just "normal" and "healthy for the baby." Yet when mothers were asked to discuss their own experience, many perceived their own breast milk as insufficient to fill an infant's nutritional needs. In this case, they perceived formula to be a good source of extra calories to help an infant grow or feel full. Mothers also suggested that the price of formula was too high to exclusively give it to their baby.

Maternal beliefs regarding the appropriate time to stop nursing ranged from 8 months to 3 years. Most mothers cited that doctors recommended nursing into the second year, and that the introduction of complementary foods should begin around the 6th month of life. Although almost all of the mothers agreed that 6 months of milk feeding was the recommendation of doctors, they thought that infants can be introduced foods much earlier (some in the 2nd and 3rd month). The term "exclusive" breastfeeding was one that I introduced, and seemed to be far from the minds of most mothers. One mother described what others echoed: “Doctors say that if you give food before that, they get sick. I gave my kids food at 3 months. They were hungry! When they watched me eat, they wanted food too. How could I not?” Most reasons given for waiting for 6 months were that infants could better digest foods at that time. One mother reasoned that she did not give her children food before 6 months “because if I gave it before, the doctors would yell at me. Don't know why, but they say that!” In fact, many of the responses to my interviews began with “well the doctors say X,” and then were finished often by a statement of agreement or disagreement.

Mothers mostly agreed that the first foods introduced to infants should be specifically formulated or processed for them. The term “gerber” has been appropriated to mean any vegetable or fruit puree (whether actually of the Gerber brand, generic or homemade). Soups made from powdered broth and a few vegetables, sweet breads dipped in coffee or milk, and rice or wheat cereals are common first foods. These foods lead the way to a typical adult diet, which consists of corn tortillas, breads (sweet and savory), corn based pupusas, soups, beans, meats, yuca, fruits and an ever-growing array of processed, energy dense “junk” foods.

Summary Statistics on Early Feeding by Infant Age



Age	Breast Fed	Mixed Fed	Formula Fed
0-3 mo. (n=36)	17	17	2
4-7 mo. (n=54)	21	26	7
8-11 mo. (n=44)	18	17	9
12+ mo. (n=50)	22	18	10

Figure 34: Summary statistics of early feeding practices among age groups.

Note again that many of the answers to these questions began with “the doctors say…” These mothers have been inundated with public health campaigns designed to teach them these “ideal” feeding and care practices. Almost all mothers are very aware of what doctors and other medical professionals advocate. Often the responses from mothers felt much like a tape recording, straight from the mouth of public health and medical personnel. As one mother admitted, “I know very well what the public health worker wants to hear. I get tired of them telling me the same thing, so I tell them what they want to hear about how I feed and care for my baby and they leave me alone. Otherwise I hear the same lecture over and over.” Indeed, public health campaigns in the area have placed workers who actively and copiously remind mothers of ideal timings for feeding and different care practices. After hearing “the doctors say,” I would ask “and what do you think about this?” Often mothers would pause, and some would say they agreed with and followed the recommendation, while others began to tell me why they diverged from the recommendations. Most commonly mothers considered reasons for divergence to be conditions outside of their own control, including their child’s preferences or demands, health concerns for child or mother, doctor’s orders, the price of alternative milk, or the perceived inability to produce adequate milk supply. The following section will more clearly detail these reasons for actual feeding practices.

Infant feeding practices

As in most places around the world, beliefs around appropriate feeding and actual practices of infant feeding diverge. In Joya de Cerén only 32% of infants are exclusively breastfed between the ages of 0-6 months. These rates match the national levels and fall only slightly behind global averages of 35% (WBTI 2015). The percentage of breastfed babies rose in the 7th month to 42%, presumably because infants were receiving complementary foods and were perceptibly less dependent on infant formula and early cereal supplements. The decrease in supplemental formula by the 7th month aligns with the choice of many mothers to supplement their breast milk with formula in the first place. Mothers often reported that they supplemented with formula or cow's milk because they produced too little milk and infants were hungry after breast-feeding. As mothers began to feed complementary foods, formula supplementation decreases, and higher rates of mothers claimed exclusive breastfeeding.

Mothers gave multiple reasons for exclusive breastfeeding: among the most prevalent were because it was healthier for the infant, the exorbitant cost of formula, and the convenience of breastfeeding. The latter reasoning was almost exclusive to women who did not have running water, for whom cleaning and preparing bottles of formula was a task. Another common reason for exclusive breastfeeding was infant distaste to formula, or preference to breast milk. A few mothers said that their infants could not tolerate formula and became ill.

When I inquired about the timing of breastfeeding, only 38% of mothers were able to initiate breast-feeding within the first hour after giving birth. Because more than half of the mothers said their infants were taken away directly after birth, this smaller percentage of mothers was able to feed their babies colostrum. The mothers who did not feed colostrum said it was because their infants were taken away for observation or procedures immediately following birth (for days to months depending on the medical concern).

The majority of mothers said that they learned about breast-feeding in the hospital or health clinic rather than from their mothers, mothers-in-law or other family members. A few participants suggested that their mothers or mother in laws were insistent on their breastfeeding and supported with instruction. One mother said that her mother was too old and had forgotten, thus was no help to her. Another mother said she had watched so many mothers breast-feed that it just came naturally and she figured it out on her own. Mothers said that they decided how much milk and when to feed by infant cues. When infants cried or fussed, they fed them. When they fell asleep, or finished eating, they ended the breast-feeding session.

Mothers gave a variety of reasons for supplementing their infants with alternative milk. Mixed feeders often replied that they gave their babies formula when the infants did not fill up on breast milk. Many mothers suggested that their milk didn't come in for a period (or at all), or they believed that they didn't produce enough milk to satisfy their baby's dietary needs. Mothers generally preferred their babies to be fat and formula supplementation helped them achieve this. One mother was losing too much weight from breastfeeding and decided to supplement her child with formula. Another couple was concerned that the mother's anger was transferred through her breast milk and would upset her child's GI tract. This mother supplemented her child with formula during her "moody" episodes.

Mothers indicated that work patterns also greatly influenced whether they could exclusively breast-feed (see Figure 36). Formal market participation meant (for all but one case) that mothers did not breast feed or pump during working hours. Instead infants were fed formula or given other alternatives during working hours. Mothers who participated in the informal market had more flexible schedules where they could nurse while working. Such mothers often were also poorer and could not afford formula, which made nursing more of an imperative. Other mothers who said they could not afford formula and yet who needed to supplement turned to bottles filled with powdered cow's milk, sugar and cereal, or other alternatives. One of the poorest families gave the infant a powdered orange drink while the mother was away at work because they could not afford infant formula and they did not have refrigeration to store expressed milk.

A total of 16% (n=11) of mothers never initiated breastfeeding. Mothers who formula-fed determined how much milk to feed their babies by the instructions on the container, or directions from doctors. Formula supplementation was most often prepared following directions on the can. Mothers boiled water, and mixed with the appropriate amount of powdered formula. When pressed, many mothers admitted to adding up to 3 teaspoons of sugar in each baby bottle. As infants grew older or mothers perceived that their kids were not gaining weight or were still hungry, mothers added instant infant cereals to bottle formulations.

Four of these mothers found the costs of formula to be too high and instead supplemented their infants with powdered cow's milk. One grandmother used orange drink to supplement the baby during the long days while the mother was away at work in a factory that did not support breast pumping. The other six mothers supplemented their infants with store-bought infant formulas. Another infant was formula fed for the first two months of life and then fed powdered cow's milk after that. Mothers of infants who were exclusively formula-fed often gave the reason that the baby had a birth complication and was hospitalized in the first days and weeks of life. In these cases, the mothers pointed to their perceived physical inability to express milk by the time the baby was in her care, or that the baby preferred the bottle to her breast-milk.

Interestingly 16% of mothers (n 11/69) began feeding their infants with formula, and then later shifted to increased or exclusive BF. Rationales for these changes included the exorbitant price of formula, the infant getting sick or becoming intolerant of cow's milk or formula (and the pediatrician suggesting the return to predominantly or exclusive breastfeeding), and more milk coming in. One mother who made this shift from predominantly formula to predominantly breastfeeding said her milk didn't fall much in the first month. She started feeding him more formula, but the formula was too expensive. Her father went to get her a certain kind of fish and her mother began feeding her a special soup with local herbs, which helped her milk come in so she could nurse more. She said it was not easy, but she worked hard to produce enough milk for her baby. Without such support from her family, she said it would not have been possible and she did not know what she would do because she could not afford formula.

This aligns with a body of literature that emphasizes the importance of family, community and medical support in helping mothers initiate and continue breastfeeding (WHO 2001; WHO 2003; Chantry 2011).

There was also variation between what mother indicated was the optimal age for initiating complementary feeding and when they actually initiated the feeding. While most mothers suggested that infants should receive commentary foods by the sixth month, 72% of mothers began giving their child foods "to taste," as early as 3-5 months, while only 26% waited until the 6th month.

Although mothers were well-aware of biomedical protocol as to timings of early feeding, actual practices differed substantially. Exclusive breast-feeding rates were reflective of international levels. Mothers found work and insufficient milk production to be major barriers to exclusive feeding, while they found the cost of formula to be the main barrier to milk supplementation.

Section II: Research questions and hypotheses

This chapter is based on the proposition that within community differences in household ecologies (shaped by broader socio-political and economic forces) will produce differential microbial exposures and therefore variation in microbiome development. The following research questions and hypotheses explore this principal framework.

H1: Child care

H1a: Childcare networks should increase as infants grow older, more independent and more mobile.

H1b: The size of childcare-networks should be positively correlated with alpha diversity.

H2: Maternal labor patterns

H2a: Infants with mothers who work should have greater horizontal exposures, and higher resulting α-diversity than infants with mothers who stay at home.

H2b: Working mothers should be more likely to mix- and formula-feed their infants than mothers who stay at home.

H2c: Infants of working mothers should be more likely to get sick, and will receive more antibiotics than infants of mothers who stay at home.

H3: Paternal care patterns

H3a: Greater maternal participation in the formal labor market should be associated with a facultative increase in paternal participation; lower SES fathers will be more likely to participate in childcare.

H4: Socioeconomic standing (SES)

H4a: Infants from the low objective SES group should have higher microbial exposures and α-diversity than infants from the high objective SES group.

H5: Household microbial ecology index (HMEI)

H5a: Infants from the low HMEI group (low sanitation/high exposure) should experience more microbial exposure, and have higher α-diversity than infants from the high HMEI group (high sanitation/low exposure).

Q5a: What microbes are guiding the differences among HMEI groups?

H5b: Middle HMEI infants should be healthier than higher or lower HMEI infants.

H5c: Infants of working mothers should be evenly distributed among the HMEI groups.

H6: Feeding ecology/Overall model

Q6a: What are the main factors that influence alpha diversity in this population?

Methods

The household unit is central to the study population and therefore this chapter. In Joya de Cerén, households are central spaces and even webs of spaces within which family and infant care practices are decided and enacted. Households are the first material environments that infants physically interface with daily. For these reasons, extensive ethnographic research was conducted in and between households. Formal and informal interviews, as well as participant observation were conducted in the infant's primary household (when safety and logistical considerations permitted). Data was collected on how infants interfaced with their caretakers and their surrounding microbial ecology, both of which presumably shape gut microbiota (Ley et al. 2008; Yatsunenko et al. 2012; Dominguez-Bello and Blaser 2011). Thus, examination of households (i.e. household construction and maintenance, ties to broader economy and community, internal social dynamics, who makes decisions and why) is critical to understanding early microbial exposures and colonization in this population. Study participants included 71 infant and primary caregiver pairs. Participants were recruited through the local public health worker. Infant participants were between the ages of 0 and 12 months at baseline (average age at baseline = 4.8

mo). Approximately one-quarter of the infants were delivered by cesarean section (see Table 15).

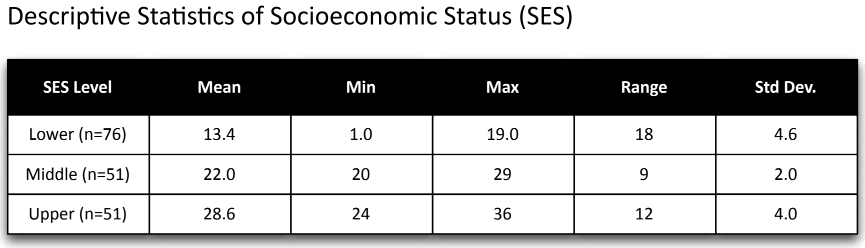
Descriptive Data of the Study Population, n = 71

Variable	Subgroup	Mean	Median	Iq Range or %
Mother's Age (yrs.)		25.3	24	(21-30)
Mother's Education		8.5	9	(6-12)
Gender	Male	38		54%
	Female	33		46%
Infant Age at Baseline (mo.)		4.8	4	(1-7)
Infant Age at 2nd Collection (mo.)		8.2	7	(5-11)
Infant Age at 3rd Collection (mo.)		12.8	12.5	(9-16)
Birth Mode	Vaginal	53		75%
	C-Section	18		25%
Birth Weight (gr)		3049.8	3005	(2797-3473)
Birth Length (cm)		48.4	49	(47-50)
Days of hospitalization		2.7	0	(0-3)
Antibiotic Administration		2.3	2	(1-3)
Diarrhea Episodes		2.8	2	(1-4)
Upper respiratory		1.8	1	(1-3)
GI		1.2	1	(0-2)
Fever		0.5	0	(0-1)
Allergy		0.2	0	0
Total Illness		3.7		
Early Feeding	Pure Breast milk (BM) (n=16)			23%
	Predominantly BM (n=28)			39%
	Mixture (n=9)			13%
	Predominantly Formula (n=8)			11%
	Pure Formula (n=10)			14%
Currently Breast Feeding	Yes (n=154 obs)			
	No (n=28 obs)			
Birth Complication	Yes (n=37)			
	No (n=34)			
Birth Timing	Premature (n=4)			
	Term (n=64)			
	Post-mature (n=4)			
HMEI Scale	Lower (n=10)			
	Middle (n=25)			
	Upper (n=23)			
SES Levels	Lower (n=28)	13.64	14	7.5
	Middle (n=19)	22.32	22	3
	Upper (n=21)	28.5	28	5

Table 15: Summary statistics of descriptive data.

Early feeding was measured in infants > 6 mo.

Over the course of one year, a minimum of three home visits and interviews with participating caregivers was conducted, during which anthropometric measurements (length and weight) and fecal samples were collected. At least three fecal samples were collected per infant, with additional sampling around key events (e.g. administration of antibiotics, severe illness, etc.). Disposable diapers were delivered to caregivers the night before a sample was collected, and soiled diapers were collected within two hours of infant defecation. Samples were collected with sterile swabs and stored at -20° Celsius for up to three months before being packaged on ice and hand-delivered to the Knight Laboratory at the University of Colorado at Boulder for sequencing.



Descriptive Statistics of Socioeconomic Status (SES)

SES Level	Mean	Min	Max	Range	Std Dev.
Lower (n=76)	13.4	1.0	19.0	18	4.6
Middle (n=51)	22.0	20	29	9	2.0
Upper (n=51)	28.6	24	36	12	4.0

Table 16: Summary statistics of objective socioeconomic standing.

This table shows basic summary statistics for each SES level. SES ranged from 0-36 on the scale and included metrics such as education, maternal work status, household status, monthly household income, and family remittance.

Bacterial DNA was extracted from the samples using the Eppendorf EpMotion 5075 robot. Polymerase chain reaction (PCR) was then used to amplify the 16S ribosomal RNA (rRNA)[4] genes to characterize the taxa present in the gut microbial communities of these infants (n = 215 samples successfully analyzed). Samples were then sequenced on

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[4] The 16S rRNA gene (often referred to as “16S”) is widely used in studies of microbial ecology as a “barcode gene” (Hebert et al. 2003) to quantify microbial community structure and diversity (Pace 1997; Hugenholtz et al. 1998). The widespread use of 16S as a barcode gene has been driven in part by the fact that it is universal across bacteria and archaea, and can be easily amplified from a wide diversity of taxa at one time by the polymerase chain reaction (PCR). For these reasons the 16S gene is phylogenetically informative, because it can be used to identify and phylotype sequences based on extensive databases of 16S sequences with associated taxonomic and phylogenetic information (Pace 1997; Woese and Fox 1977). There are numerous advantages to using 16S as a microbial community barcode gene, but also numerous disadvantages including amplification and sequencing bias and error (Suzuki and Giovannoni 1996; Hong et al. 2009), difficulty with the accurate taxonomic identification and binning of short sequences (Qin et al. 2010; Kunin et al. 2010; Haas et al. 2011; Rodrigue et al. 2010).

the Illumina second generation sequencer. Alpha diversity[5] (α-diversity) was calculated using Chao1 diversity index based on the rarefied OTU table at a depth of 1226 sequences per sample. The calculations were performed either with the QIIME pipeline (Caporaso et al., 2010), with R software (R Foundation for Statistical Computing, Vienna, Austria) using the vegan (Oksanen et al., 2011) and picante (Kembel et al., 2010) packages, and/or JMP Pro 12 software. To compare between-sample variations in the composition of the total microbial community, unweighted and weighted UniFrac distances (Lozupone and Knight, 2005) were calculated with the QIIME pipeline (Caporaso et al., 2010). PCoA (Principal Coordinate Analysis) was performed on both distance matrices and coordinates were used to draw 3D graphical outputs.

For analysis, the age variable was used both as a continuous factor by month and a grouped-age variable (0-3 months, 4-7 months, 8-11 months, and 12+ months). These age groups were determined based on common life stages that occur around chronological transitions appropriate for this population. Infants 0-3 months largely received milk substrates, whereas mothers in this area tend to introduce "taste" complementary foods around the 4th month of life. Thus the 4-7 months group captured the transition to complementary feeding. The 8-11 months group is a transitional group where infants are often still receiving milk

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[5] Alpha diversity is a summary measure of intra-sample taxonomic diversity, which captures both the organismal richness of the sample and the evenness of the organisms' abundance distribution.

(either formula or breast), and 12+ months is commonly a time when infants will be weaned off of breast milk or bottle.

Results

H1a: *Childcare networks should increase as infants grow older, more independent and more mobile.*

The raw number of individuals in a care network did not significantly change with child age, although there was a trend in this direction (F=3.5, P=.06), based on one-way ANOVA. A range of family relations made up these networks, including grandparents, aunts and uncles, siblings, cousins, and in a few cases, friends, neighbors, and/or paid caretakers. Interestingly, care networks also tended to increase with socioeconomic (SES) level, although also not significantly (F=3.7, P=.055).

H1b*: The size of childcare-networks should be positively correlated with alpha diversity.*

To investigate whether the size of care networks would positively influence infant alpha diversity, a standard multiple regression was performed. A significant regression equation was found (F= 205.8, P= .0001), with an R^2 of .07. As care networks increase, so did alpha diversity (P=.001), even after controlling for the confounding effects of age on alpha diversity (P=.001). The augmentation in alpha diversity is likely due to the increased horizontal exposure from multiple caretakers, differential care-behaviors, formula-feeding, and multiple household ecologies).

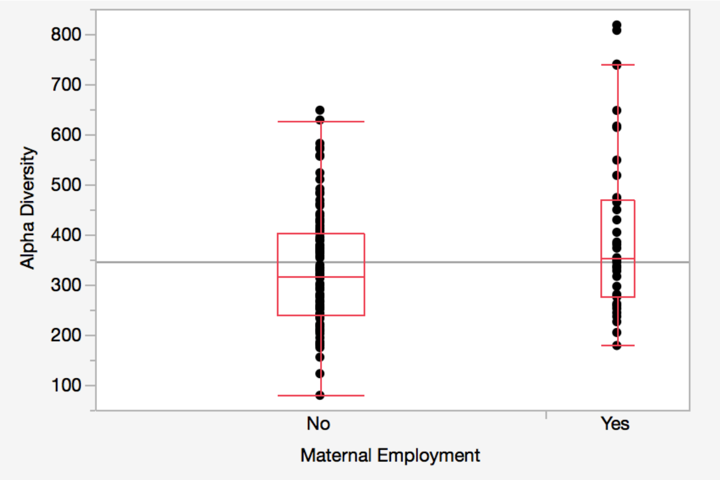


Figure 35: A-diversity and maternal employment.

infants of mothers who worked had higher α-diversity than those of mothers who stayed at home.

***H2a**: Infants with mothers who work should have greater horizontal exposures, and higher resulting α-diversity than infants with mothers who stay at home.*

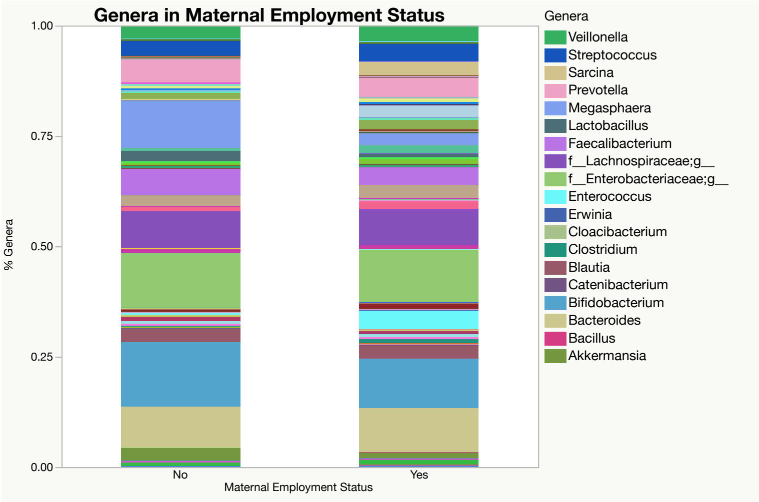
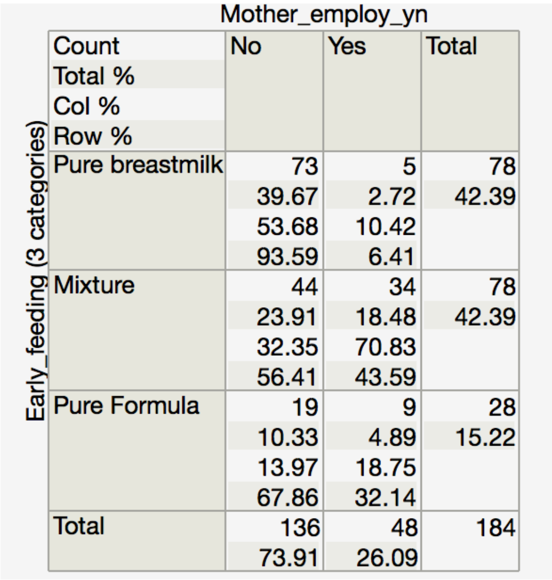


Table 17: 100% stacked bar graph of bacterial taxonomic composition among infants of working mothers vs. stay-at-home mothers.

Infants of working mothers have more diverse, less stable and more inflammatory microbiomes. Yes= employed, No= stay-at-home.

Maternal employment is significantly related to α-diversity (F=11.2, P=.001) even after controlling for infant age, as determined by one-way ANOVA. Infants of mothers who worked had significantly higher α-diversity than those of mothers who did not work (see Figure 35). Moreover, infants of working mothers had significantly more *Blautia* (F=5.3, P=.02) and Lachnospiraceae (F=4.3, P=.04), and less *Bifidobacteria* (F=5, P=.02) than infants of mothers who stayed home. Higher early α-diversity, with higher levels of inflammatory bacteria such as

Blautia and Lachnospiraceae, in addition to lower levels of important *Bifidobacterium,* indicates that infants of working mothers harbored an unstable, dysbiotic microbiome (see Table 17).



		Mother_employ_yn		
Early_feeding (3 categories)	Count Total % Col % Row %	No	Yes	Total
	Pure breastmilk	73 39.67 53.68 93.59	5 2.72 10.42 6.41	78 42.39
	Mixture	44 23.91 32.35 56.41	34 18.48 70.83 43.59	78 42.39
	Pure Formula	19 10.33 13.97 67.86	9 4.89 18.75 32.14	28 15.22
	Total	136 73.91	48 26.09	184

Table 18: Contingency table of early feeding behaviors among working and unemployed mothers.

Infants of working mothers tend to be mixed fed (70.83%). 93% of the exclusively breast-fed group had mothers that stayed at home.

***H2b**: Working mothers should be more likely to formula-feed their infants than mothers who stay at home.*

Only 6.4% of working mothers exclusively breast-fed their infants compared to unemployed mothers. Of working mothers, 71% mixed-fed their infants, while 19% exclusively formula-fed. Conversely, 54% of stay-at-home mothers exclusively breast-fed, making up 94% of the exclusively breast-fed group (see Table 18), indicating that rates of exclusive breastfeeding may be substantially higher if women were supported with programs at work and longer maternal leave, etc.

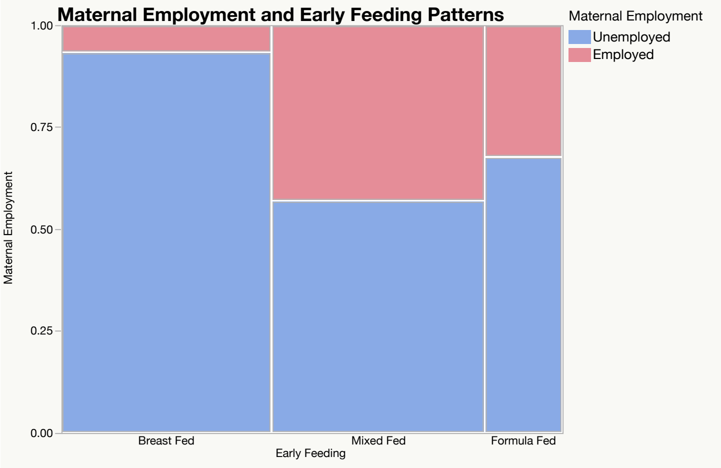
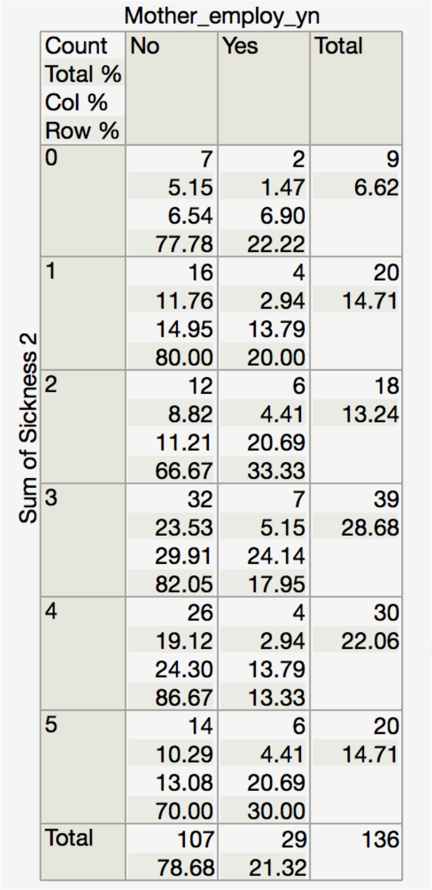


Figure 36: Maternal employment and early feeding patterns.

Infants of mothers who worked were more likely to mix-feed or to exclusively formula-feed their infants.

These analyses underscored that infants of working mothers in this population are primarily mixed-feeders.

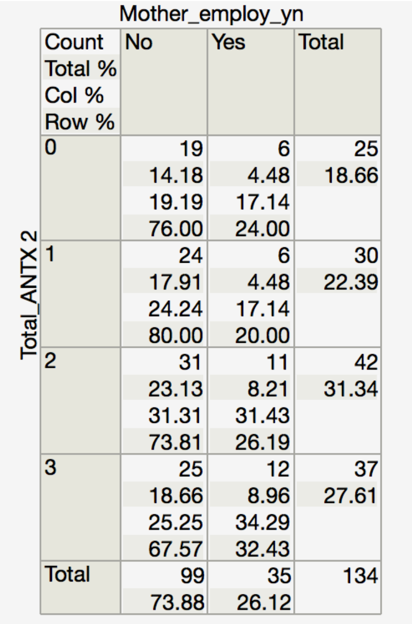
***H2c**: Infants of working mothers should be more likely to get sick, and will receive more antibiotics than infants of mothers who stay at home.*



Sum of Sickness 2	Mother_employ_yn		
Count Total % Col % Row %	No	Yes	Total
0	7	2	9
	5.15	1.47	6.62
	6.54	6.90	
	77.78	22.22	
1	16	4	20
	11.76	2.94	14.71
	14.95	13.79	
	80.00	20.00	
2	12	6	18
	8.82	4.41	13.24
	11.21	20.69	
	66.67	33.33	
3	32	7	39
	23.53	5.15	28.68
	29.91	24.14	
	82.05	17.95	
4	26	4	30
	19.12	2.94	22.06
	24.30	13.79	
	86.67	13.33	
5	14	6	20
	10.29	4.41	14.71
	13.08	20.69	
	70.00	30.00	
Total	107	29	136
	78.68	21.32	

Table 20: Contingency table of total illness among infants of working and unemployed mothers.

Working mothers only reported 21% of the total illnesses reported, yet make up ~28% of overall population.



Total_ANTX 2	Mother_employ_yn		
Count Total % Col % Row %	No	Yes	Total
0	19	6	25
	14.18	4.48	18.66
	19.19	17.14	
	76.00	24.00	
1	24	6	30
	17.91	4.48	22.39
	24.24	17.14	
	80.00	20.00	
2	31	11	42
	23.13	8.21	31.34
	31.31	31.43	
	73.81	26.19	
3	25	12	37
	18.66	8.96	27.61
	25.25	34.29	
	67.57	32.43	
Total	99	35	134
	73.88	26.12	

Table 19: Contingency table of infant antibiotic loads among Infants of working mothers.

Infants of working mothers are less likely to receive antibiotics.

Working mothers reported only 21% of the total illnesses reported and made up 28% of the participants. Infants of working mothers received 26% of the total antibiotics consumed by the study population. This is a surprising outcome, considering that mixed-fed infants and infants of working mothers in particular had higher α-diversity, and less stable microbiomes. However, working conditions in this region may help explain these low rates of reporting illness and antibiotic administration. Factory workers in this region work 12 hour days, 6 days a week. Working mothers do not have the time to take their infants to the doctor, and it is likely that caretakers are less concerned with infant illness than mothers who are primary care-takers in the study. It is also possible that working mothers who do not spend as much time with their infants are less aware of the illnesses their infants experience.

***H3a**: Greater maternal participation in the formal labor market should be associated with a facultative increase in paternal participation; lower SES fathers will be more likely to participate in childcare.*

Conversely, socioeconomic status (SES) was positively correlated with the maternal perception of paternal infant care participation (F= 3, P=.03), showing that in opposition to **H3a**, that women of higher SES perceived their partners to be more involved in childcare. This positive maternal perception may be bolstered by economic contribution from fathers, or it is possible that higher SES fathers are simply more participatory in childcare. Unfortunately, this measure was assessed solely as maternal perceptions of paternal participation in child care, not providing time allocations or specific care activities.

***H4a**: Infants from the low SES group should have higher microbial exposures and α-diversity than infants from the high SES group.*

Although subjective SES was not significantly related to α-diversity, SES was inversely associated with α-diversity levels (F=3.53, P=.032). The significance is only found between the upper and lower SES levels (Tukey pairwise comparison p= .034). Thus lower SES levels have significantly higher α-diversity than high SES individuals.

H5a: *Infants from the low HMEI group (low sanitation/high exposure) should experience more microbial exposure, and have higher α-diversity than infants from the high HMEI group (high sanitation/low exposure).*

Descriptive Statistics of Household Microbial Ecology Index (HMEI)

HMEI level	N	a-diversity
Lower	26	392.38
Middle	63	358.87
Upper	62	300.07

Table 21: A-diversity among HMEI groups.

The mean alpha diversity of the low HMEI group is significantly higher than the group of infants who are in the high HMEI group.

HMEI is significantly and inversely related to α-diversity, independent of age (F=9.5, P=.0001). The high HEMI group (high sanitation/low exposure) has significantly lower α-diversity compared to the middle HMEI group (p=.007) and the low HMEI group (low sanitation/high exposure) has significantly higher α-diversity than the high HMEI group (p=.0003) (see Table 21 and Figure 37 Figure 38). Figure 35 shows that low HMEI infants tend to acquire α-diversity at a faster pace than either middle or high HMEI infants.

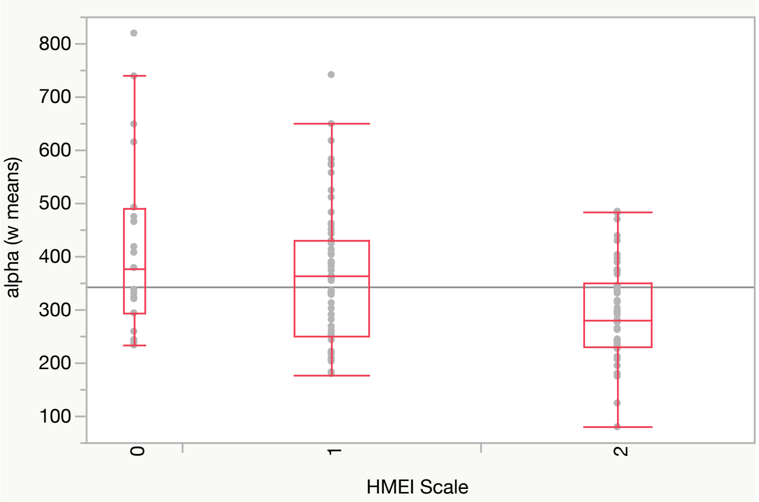


Figure 37: Levels of α-diversity among the HEMI levels.

This boxplot shows the different levels of alpha diversity in the three levels of HMEI scale. 0 represents the low HMEI group, 1 is the middle group and 2 is the high HMEI group.

***Q5a:** What microbes are guiding the differences among HMEI groups?*

High HMEI infants harbored the highest levels of *Bifidobacterium*, *Blautia*, *Enterococcus*, and lowest levels of Enterobacteriaceae, and *Staphylococcus*. Middle HMEI infants harbored the highest levels of *Bacteroides*, *Faecalibacterium*, *Lactobacillus*, *Megasphaera*, *Roseburia*, *Ruminococcus*, *Veillonella*, and the lowest levels of *Akkermansia*, *Lachnospiraceae*, and *Streptococcus*. Finally, Low HMEI infants harbor the highest levels of *Akkermansia*, *Clostridiales*, Enterobacteriaceae, *Prevotella*, and the lowest *Roseburia*. In summary, middle HMEI infants harbor higher levels of many anti-inflammatory, immune-protective bacteria including *Lactobacillus*, *Faecalibacterium*, and *Roseburia*, and bacteria that are known

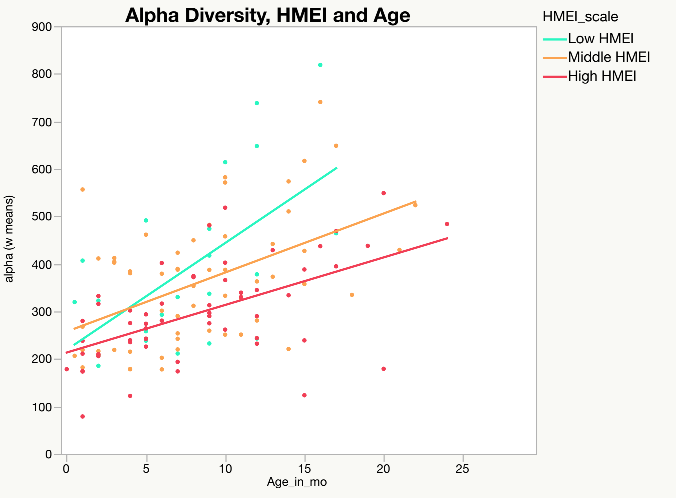


Figure 38: A-diversity, HMEI and age.

This graph shows that in this population, low HMEI infants tend to acquire diversity more rapidly than middle or high HMEI infants, and that these differences are more pronounced at 12+ mo.

HMEI	**Low**	**Middle**	**High**
N Rows	26	63	62
Akkermansia	3.56%	0.56%	2.77%
Bifidobacterium	12.08%	15.75%	18.09%
Bacteroides	4.13%	9.68%	8.42%
Blautia	2.75%	1.52%	4.06%
Clostridiales	1.27%	0.66%	0.65%
Enterobacteriaceae	25.70%	20.55%	17.55%
Enterococcus	0.40%	0.25%	1.59%
Faecalibacterium	2.01%	2.61%	1.63%
Lachnospiraceae	6.83%	5.25%	6.61%
Lactobacillus	2.23%	3.94%	2.25%
Megasphaera	5.87%	8.74%	4.76%
Prevotella	4.27%	2.54%	0.70%
Roseburia	0.01%	0.06%	0.02%
Ruminococcus	0.83%	0.96%	0.60%
Ruminococcaceae	1.35%	1.63%	1.00%
Staphylococcus	0.14%	0.10%	0.05%
Streptococcus	7.06%	6.52%	7.67%
Veillonella	3.77%	5.23%	5.20%

Table 22: Relative abundances of the main bacteria among the HMEI levels.

fermenters of breast-milk including *Bacteroides, Lactobacillus, Megasphaera,* and *Veillonella*. Notably, middle HMEI infants were found to harbor significantly higher levels of Lactobacillus (F=4.9, P=.009) compared to infants of the low (P=.028) or high HMEI group (P=.032). This finding was important because of the low-prevalence of this critical and beneficial microbe in the general population. Higher prevalence in this group is likely due to the microbe's association with breastfeeding and optimal early exposures. Meanwhile middle HMEI infants harbor the lowest levels of more inflammatory microbes including Lachnospiraceae and *Streptococcus*. Low HMEI infants harbor the highest levels of the most inflammatory microbes including *Clostridiales*, Enterobacteriaceae and *Prevotella*. For relative abundances, see Table 22 and Table 23. Based on the relative abundances of these significant bacteria, the middle HMEI group would appear be the healthiest group of infants. While this study only explores

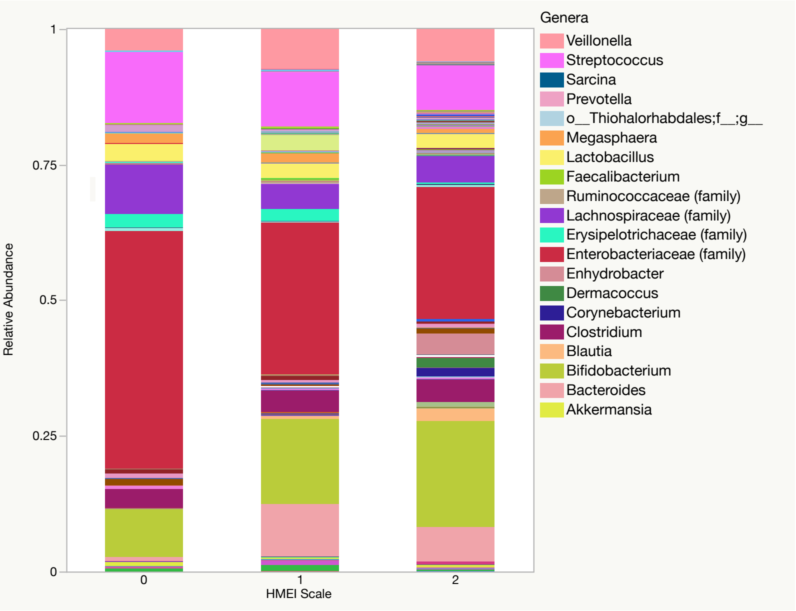
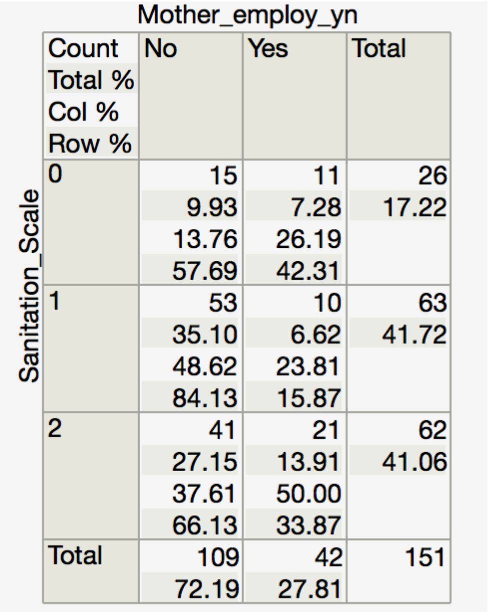


Table 23: 100% stacked bar graph of bacterial taxonomic composition among infants of the three levels of HMEI.

0 = low HMEI group (high exposure), 1= middle HMEI, 2= high HMEI (low exposure). The above graph illustrates the difference in certain important genera including higher levels of Enterobacteriace, Streptococcus, Lachnospiraceae among infants in the lower HMEI group, and higher levels of Bifidobacterium, Bacteroides, and Blautia among high HMEI infants. Moreover high HMEI infants had increased levels of Enhydrobacter, Dermacoccus and Corynebacterium which are commonly seen on the skin of adults (Ling et al. 2013).

microbial diversity within one population, it would be interesting to adapt the HMEI measure to use in cross-cultural comparisons.



Sanitation_Scale	Mother_employ_yn Count Total % Col % Row %	No	Yes	Total
Sanitation_Scale	0	15 9.93 13.76 57.69	11 7.28 26.19 42.31	26 17.22
	1	53 35.10 48.62 84.13	10 6.62 23.81 15.87	63 41.72
	2	41 27.15 37.61 66.13	21 13.91 50.00 33.87	62 41.06
	Total	109 72.19	42 27.81	151

Table 24: Contingency table of HMEI and maternal employment.

Infants of working mothers are most likely to make up the low or high HMEI group.

H5b: *Middle HMEI infants should be healthier than higher or lower HMEI infants.*

To test **H4b**, a chi-square test was performed on various indicators of health. Oddly, the percentage of middle HMEI infants who took 3+ antibiotics was significantly more than the other two groups $c^2(6, N = 116) = 0.03, p = .02$. However, the low HMEI group was more likely to experience 6+ total illnesses over the course of the study period, $c^2(12, N = 149) = 0.001, p = .0001$. This may be explained by mothers from the low HMEI group being less concerned about an illness, taking the child to the doctor less frequently, receiving fewer doses of antibiotics, yet the child having recurring infections because of the higher than average environmental exposures. Seeing that mothers from the low SES group tend to make up more of the low HMEI group, it is likely that these mothers have time and financial constraints that prevent them from taking their sick infants to a clinic visit. These findings indicate that middle HMEI infants may be the healthiest of the groups, however receive the highest dosages of antibiotics, while low HMEI infants do get sick more often, yet receive the fewest dosages of antibiotics.

H5c: *Working mothers should be evenly distributed among the HMEI groups.*

Working mothers were most likely to be in the high or low HMEI group. Working mothers (~28% of the population) only made up 16% of the middle HMEI group, whereas they made up 42% of the low HMEI group and 34% of the high HMEI group. Of working mothers, 50% were in the high HMEI group while the remaining mothers were equally distributed among the low and middle HMEI groups (see Table 24).

Q6a*: What are the main factors that influence alpha diversity in this population?*

To explore **Q5a**, a standard multiple regression was performed. Preliminary analyses were performed to ensure there is no violation of the assumption of normality, linearity and multicollinearity. A significant regression equation was found (F= 9.6, P= .0001), with an R^2 of .49. Age in months (F=24.43, P=<.0001), maternal employment (F=9.8 P=.002), HMEI (F=9.38, P=.0002), early feeding (as an interaction with age) (F=2.65, P=.036) were all found to be significant factors to alpha diversity. Birth mode was interestingly not significant in this model even as an interaction term with age.

Further analyses determined that when controlling for age, the introduction of complementary foods did not seem to significantly alter alpha diversity. Surprisingly, the cessation of breastfeeding also did not significantly alter alpha diversity, however whether a mother is currently breastfeeding was significantly related to changes in α-diversity: infants who were currently being breastfed had a lower average diversity than infants who were not (P=.02, mean = 327.2 and 388.0, respectively). After controlling for age, this significance shows up only at 4-7 month (F=4.55, P=.038).

Discussion

Sweeping socio-political and economic changes in El Salvador have introduced massive transformations in labor markets, demographics, social networks, gender relations, and household-level socioeconomics. Young women and particularly young mothers bear the brunt of these changes. Factories in this region only hire women of child-bearing years, and yet these jobs are highly inflexible, with long hours and low pay. Most factories do not provide lactation programs, forcing mothers to supplement infants with formula or less-nutritionally adequate, yet more affordable solutions. In many cases, mothers leave factory jobs because their monthly salary is insufficient to cover the costs of formula and childcare. These mothers report that the pay is not worth their time, and instead find informal market jobs, or subsist on familial support. Other mothers continue working and face difficult decisions on what to feed their infants and how to care for them during the long hours away. Although there seems to be an increase in father participation, still 20% of the study households are single-mothers. No matter the situation, these broader changes in labor, social networks, and household dynamics alter the way that infants are fed and cared for. Infant care and feeding practices influence early exposures and likely infant microbiota during the first years of an infant's life.

This chapter utilized a developmental ecology framework to explore the effects of these broader social transformations on infant microbial ecology within this Salvadoran population. The over-arching hypothesis of this chapter was that differences in household ecologies (shaped by broader socio-political and economic forces) would produce differential exposures and therefore variation in microbiome development. A set of hypotheses were tested and explored with both expected and unexpected results.

Infant-care network size was found to be positively correlated with α-diversity. Logically, as the number of caretakers increase, so too does a suite of other exposures, from feeding and care practices to distinct household ecologies. In other words, increasing caretakers can increase horizontal exposures. The size of infant care networks was also influenced by socioeconomics: lower SES women tended to have smaller childcare networks and reported less paternal support. Of the lowest SES level, a startling 41% were single mothers. Interestingly, women of higher SES perceived their partners to be more helpful. This positive maternal perception may be bolstered by economic contribution from fathers, or it is possible that higher SES fathers are simply more participatory in childcare. Either way, these findings suggest that the poorest mothers had the least support for childcare from both care networks and fathers.

Maternal employment also significantly influenced α-diversity, and was intimately tied to household socioeconomics. Infants of employed mothers harbored higher α-diversity. This hypothesis was further supported by the finding that the bacteria driving these changes in α-diversity were significantly different: children of working mothers harbored significantly more inflammatory bacteria including *Blautia* and Lachnospiraceae, and lower levels of key probiotic bacteria such as *Bifidobacterium*. This finding indicates that the early increase in α-diversity may not be beneficial in the first months of life.

The mechanisms by which maternal employment affects young children are likely complex and multifaceted. Time limitation is seen as a major constraint on caretaking of young children and can affect decisions about early feeding practices (Chatterji and Frick 2005; Hawkins et al. 2007; Hirani and Karmaliani 2013; Lakati, Binns, and Stevenson 2002). When mothers are short on time, they are less likely to breastfeed, often introduce complementary foods earlier, and are more likely to select foods based on convenience rather than nutrition (Chatterji and Frick 2005). Mothers in this study who work were more likely to exclusively formula feed or mix-feed than those who did not work (chi-square 30.11, 2 DF, p=.0001).

Maternal employment in this study clearly had an inhibitory effect on exclusive breastfeeding. Most working mothers (71%) chose to mix-feed, with caretakers feeding the infant with formula during the day, and the mother nursing the infant at night. The fact that the majority (54%) of stay-at-home mothers exclusively breast-fed their infants may suggest that with adequate support at home and at work, higher rates of mothers would exclusively breast-feed given the chance. Interestingly, working mothers reported far fewer illnesses and their infants received fewer doses of antibiotics than did infants of stay-at-home mothers. Working conditions in this region may help explain these low rates of reporting illness and antibiotic administration. The excruciatingly long workweek does not leave time for mothers to take infants to the clinic. Working mothers do not experience their child's illness as much as stay-at-home moms, who are more likely to recall illnesses at interviews. Finally, caretakers are possibly less concerned about infant illness and less likely to spend the day waiting at the health clinic for the infant to be seen. The limitations in ascertainment of true illness severity and occurrence leave this question unanswered and for future research.

The Household Microbial Ecology Index was a useful tool for assessing early exposures in relation to microbiome development. Low HMEI infants were found to have higher levels of α-diversity than the middle HMEI group. There was also a positive significance between middle and upper HMEI groups. Middle HMEI infants were found to harbor higher levels of many anti-inflammatory, protective bacteria including *Lactobacillus*, *Faecalibacterium*, and *Roseburia*, and bacteria that are known fermenters of breast-milk including *Bacteroides, Lactobacillus, Megasphaera,* and *Veillonella*. Meanwhile middle HMEI infants harbor the lowest levels of more inflammatory microbes including Lachnospiraceae and *Streptococcus*. Low HMEI infants harbor the highest levels of the most inflammatory microbes including *Clostridiales*, Enterobacteriaceae and *Prevotella*.

While middle HMEI infants received more doses of antibiotics, the low HMEI infants experienced more reported illness. It is possible that mothers from the low HMEI group were generally less cued in to or concerned taking their infants to the doctors despite higher reported illnesses. Low HMEI households commonly had more animals in the house, were overall messier, and had higher population density. Infants were often left to play by themselves or with older siblings. These mothers likely were less concerned about infant illnesses, possibly less likely to take infants to the doctor or clinic. Conversely, infants of middle HMEI households were sick less often than lower HMEI infants and yet were given more antibiotics. Mothers of these infants likely had more time with their infants, were more cued into illnesses and more likely to take their infants to the clinic when ill, hence the higher doses of antibiotics. Additionally, working mothers were more likely to be in either the high or low HMEI groups, and these mothers did not have time for clinic visits.

Colonization patterns among HMEI groups was also interesting. Middle HMEI infants harbored the highest levels of important microbes *Lactobacillus* and *Faecalibacterium*, among the three groups. Relative abundances of these bacteria were n-shaped, as they were lower in both the low and high HMEI groups. Conversely, *Blautia* and Enterococcus were of lowest abundance in the middle group and higher in the low and high HMEI groups, illustrating a u-shaped pattern of abundance. *Prevotella* was the highest in the low HMEI group due to the high horizontal exposures, and decreased steadily in the middle and high HMEI groups. Overall these findings suggest that the low and high HMEI groups harbor higher levels of inflammatory and pathogenic bacteria, while the middle HMEI group harbor higher levels of anti-inflammatory and beneficial microbes. Additionally, this middle group harbored higher levels of breastmilk fermenting bacteria. Associations between HMEI and growth patterns are addressed in chapter 4, but in general these findings indicate that the middle HMEI infants may be the healthiest of the three groups, even in spite of higher levels of antibiotic exposures. While the low HMEI may have too much exposure for infant immunity, the high HMEI group likely did not receive enough exposure due to overly-clean households. These two groups were also less likely to be breastfed, thus not receiving maternal immune properties and microbiota. Together these findings point to the importance of early exposures in not only the colonization patterns, but also current and future health of study participants.

This chapter explored social, political economic and ecological factors from the perspective of the developing microbiome. It utilized a developmental ecology approach to examine these complex and intertwined variables and how they influence early exposures. Gendered labor patterns, community and household demographics, socioeconomics, household ecologies and early feeding patterns proved to be the most significant factors influencing these early exposures and thus infant microbial composition. This chapter illustrates the importance of incorporating a mixed-method, theory-based, developmental ecology framework in the investigation of the developing microbiome.

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CHAPTER 4: INFANT GROWTH, HEALTH AND THE GUT MICROBIOME

Introduction

The importance of early life experiences in shaping long-term risk of metabolic disorders is widely recognized within the fields of human biology and public health (Benyshek 2007; Kuzawa and Quinn 2009). A growing body of research documents how environmental factors operating prenatally can alter the occurrence of chronic disease throughout the life course (Barker 2004; Gillman 2005), and several systematic reviews have highlighted the importance of postnatal growth in understanding these chronic disorders (Baird et al. 2005; Parsons et al. 1999). Infants ranking at the higher ends of the weight or BMI distribution, and those gaining weight most rapidly have an increased risk of obesity in childhood, adolescence and adulthood (Baird 2005; Ekelund et al. 2007; Demerath et al. 2012) and elevated levels of metabolic risk factors, including blood pressure, insulin, cholesterol, and triglycerides, even when controlling for current adiposity, birthweight, childhood growth patterns, and maternal characteristics (Ekelund et al. 2007). Conversely, infants who are acutely or chronically malnourished have an increased risk of compromised immunity (Chandra 1997), impaired growth and stunting (Hizli et al. 2007; Prendergast and Humphrey 2014), cognitive impairment (Joseph et al. 2014), productivity and human capital (Martorell et al. 2010), and also increased risks of long-term metabolic disorders (Kuzawa et al. 2007; Barker 1990). These findings highlight the importance of viewing and utilizing early growth patterns as a marker of current and future health potentialities. However, understandings of the underlying causes and consequences of early growth patterns have only recently begun to be elaborated.

Recent interest has surged around the possible role of the intestinal microbiome as a potential contributor to infant malnutrition, adult obesity and other metabolic conditions (Smith et al. 2014; Bäckhed 2012; Tehrani et al. 2012; Harris et al. 2012; Devaraj et al. 2013). Human intestinal microbiota have been shown to play an important role in host energy balance and adiposity through a number of intertwined metabolic pathways (Bäckhed 2010). These include but are not limited to, the processing of otherwise indigestible polysaccharides into simple sugars and short-chain fatty acids (Guarner and Malagelada 2003), amino acid metabolism and xenobiotic biodegradation (Qin et al. 2010), increased energy harvest and storage (Turnbaugh et al. 2006), and hormonal signaling (Blaser 2014). This chapter reasons that given the metabolic capabilities of specific microbes and certain communities (Blaut and Clavel 2007; Cani and Delzenne 2007), the developing microbiome evolved in part to support the high energetic demands for growth of the human infant during the early months of life. In contrast, disrupted colonization patterns during early infancy should be reflected in divergent patterns of infant growth.

To explore the theoretical basis of these issues, this chapter draws from a rich domain within biological anthropology called comparative human biology. This perspective offers an interdisciplinary, comparative framework that examines the basis of human similarities and differences as well as the pathways to and consequences of this biological diversity (LCHB 2016). Because human biological adaptation and variation arise as a process over time, and in relation socio-ecological circumstances (Worthman 1999), this perspective focuses on the interactive feedback loops between environment and biology, particularly during early developmental periods. It is through this multi-level framework that comparisons of distinct species, populations or subpopulations, as well as across different time scales (acute, developmental, evolutionary), can reveal otherwise invisible sources of human variation, uniqueness and capacity (LCHB 2016).

A key principle utilized by human biologists is life history theory (LHT) (Stearns 1992; Charnov 1993; Kuzawa and Bragg 2012). This perspective compares the strategies of individuals and groups as they allocate the scarce resources of time and energy, between the critically important and nearly mutually exclusive processes of growth, maintenance, and reproduction. Understanding the selective pressures and tradeoffs that infants face in the first months and years of life can help illuminate co-evolutionary properties and functions of the microbiome. For example, rapid growth requires high energy availability, which may be generated by lowering other maintenance costs, or by increasing energy intake. As detailed later in the chapter, the first months of life require enormous amounts of energy to sustain a growing brain and body. I examine the life history constraints and trade-offs during early human development to investigate how rapid growth during this period is aided by an evolutionarily selected gut microbiome. Moreover, perturbations in growth during key developmental periods can have life-long metabolic consequences (Cox et al. 2014). This chapter draws from this multi-level, comparative framework to illuminate the energetic demands of human growth in the first year of life. In doing so, I explore how functions of the microbiome may have evolved to under selective pressures of host developmental demands during this early window of human growth.

This chapter is organized into two main sections. Section I outlines hypotheses about the microbiome and the adaptive challenges it likely evolved to resolve based upon the biological constraints that informed the evolution of human growth and development. Specifically, I examine early energetic demands for growth and maintenance. I then describe possible growth tradeoffs associated with high disease burden and immune development in the first year of life. Next, I outline current understandings of the interactions between the gut microbiome, early growth phenotypes, and health outcomes. From the application of this theoretical framework, I propose testable hypotheses. Section II draws from a case study of a Salvadoran population of infants to test the hypotheses generated in Section I.

Section I: Human Growth, Development, and the Infant Gut Microbiome

Selective pressures on human infant growth

In life history terms, human infants are highly altricial. Therefore, the postnatal period is when the most rapid changes to body and brain composition and take place. For example, infants double their birthweight in the first four or five months, followed by a slower quadrupling of birth weight by 24 months (Marques et al. 2004; WHO MGRS 2016). Although development generally follows a distinctive set of early growth curves, growth patterns are far from universal. Growth trajectories of breast-fed infants deviate from that of formula-fed infants. After 3 months of life, breast-fed infants grow more slowly than formula-fed infants (Dewey et al. 1995). Evaluating body composition, Butte et al. (2000) suggested that fat-free mass (including organ, lean muscle and tissue) and overall weight gain were lower in breast-fed infants compared to formula-fed infants at 3, 6 and 9 months of age.

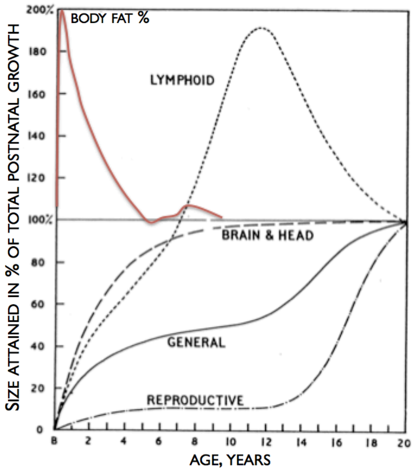


Figure 39: Body composition during the first year of life.

Nestle Nutrition Workshop Series. Paediatric Programme, 58, 65-76; discussion 76-68. adapted from Rigo, J. (2006).

Curiously, human infants deposit more fat than any other mammal. Newborns have a fat mass roughly four times that predicted for a mammal of their body size at birth (Kuzawa 1998). Estimates of body composition in well-nourished populations show that roughly 40–65% of total body weight gain during the first 4–6 months of life is accounted for by fat deposition (Fomon et al. 1982; Davies 1992), and by 6-9 months of age, infants typically reached a postnatal adiposity peak (Fomon et al. 1982) (see Figure 39). Importantly, the concentration of this energetic burden is highest during the immediate postnatal period, and fat deposition accounts for most of the total cost of growth during early infancy. It has been estimated that in the first six months of life, 72% of the approximately 20,000 Kcal necessary for tissue formation is accounted for by lipid deposition, with the remaining 28% spent on digestion, synthesis, and deposition of protein in lean tissue (Kuzawa 1998; Fomon et al. 1982; Roberts and Young 1988). The investment in fat development then gradually decreases as such fat percentages begin to decline, eventually reaching a pre-pubertal trough between 5 and 7 years of age to roughly 13% for males and 16% for females in early adulthood (Kuzawa 1998; Fomon et al. 1982).

Infants who have reduced fat stores due to prematurity, nutritional deprivation, and/or high disease burden will attempt to catch up to their healthy peers, given nutritional availability. Premature newborns often have reduced fat stores at birth for their gestational age, but experience a rapid pace of postnatal fat deposition. These infants can normalize their BMI (relative to reference data) by 6 months of age (de Gamarra et al. 1987; Micheli et al. 1994). Newborns who were born lean for unknown reasons, also tend to put on more fat than their initially fatter peers, leading to a convergence in adiposity after several postnatal months (Garn et al. 1956; Davies 1980). This catchup growth highlights the seemingly important and evolutionarily conserved trait among humans for fat deposition in the early months of life.

Researchers have long speculated about the evolutionary purpose of the relatively high rates of early fat storage in human infants. Some have suggested that energy stores are utilized as a buffer for the high burden of infection experienced during the early months and years (Prentice and Darboe 2008; Wells 2010). In fact, peak adiposity is reached at an age that complementary feeding usually begins, a developmental phase that is associated with higher risk of malnutrition and infection (Prentice and Darboe 2008). Others show that fat deposition as energy stores may be mobilized during infancy and the first years of life for the development of lean mass. Skinfold-thickness measurements during infancy are positively associated with relative amounts of childhood lean mass growth (Wells 2010), indicating that infant energy stores are used in part to meet the costs of growth. The accretion of lean tissue is a relatively slow process, requiring energy for both its construction and its maintenance (Wells 2010). Finally, human biologist Christopher Kuzawa (1998) hypothesized that the accumulation of body fat during infancy can be attributed to another uniquely human trait: the encephalized brain. In a comprehensive review of the literature, Kuzawa suggests that high rates of infant adiposity evolved to support the maintenance and growth of a relatively large brain during infancy and the weaning period. Moreover, additional energy stores in the infant body can buffer the brain's demands against disruptions in energy supply.

Indeed, compared to our closest primate relatives, human infants are born with large brains that grow quickly during infancy and are energetically expensive to maintain. The human newborn, for example, uses 87% of its resting metabolic rate (RMR) for brain growth and maintenance, compared to only 45% RMR value for the chimpanzee neonate (Bogin 2010). By the age of 5 years, the percent of RMR usage in humans is still high, at 44%, compared to 20% in chimps (ibid). Finally, adult human brains only utilize between 20% and 25% of RMR, whereas adult chimpanzees use no more than 9% at adulthood (Leonard and Robertson 1994; Bogin 2010). In the first three months of life, the human infant's brain grows to nearly 55% of its final size (Holland et al. 2014). More than one-third of the change in brain architecture in the first six postnatal years happens within the first six months, and 8 percent of the change occurs in the first month (Shankle et al. 1998).

Interestingly, only a small proportion of the energetic burden is allotted to brain growth (Martin 1981), but rather much of the cost is allocated to cerebral metabolic maintenance (E. Armstrong 1983). Most of the mass of the brain is water, with fat and protein accounting for only about 9.4% (44.5 g) and 10.1% (47.7 g), respectively, of brain growth during the first postnatal year. This represents only 3% of the 1,740 g of lipid and 6% of the 804 g of protein deposited in the growing infant body between birth and 12 months of age (male/ female average from Fomon et al. 1982). This suggests that the amount of lipids required for brain growth is minimal compared to body lipid deposition (Kuzawa 1998), and thus a majority of energy used by the brain is for maintenance rather than growth.

The human infant brain is not only expensive to maintain, but it is also particularly inflexible in its demands. The brain's minimal capacity to shrink during starvation (Kuzawa 1998), suggests that energy is necessarily diverted from other sources during a negative energy balance. Human infancy is therefore a life stage when the constraints imposed by energetic challenges of somatic growth and inflexibility imposed by brain size, are particularly acute. In fact, fat stores are laid down precisely at the same time when energy requirements for growth are at their maximum. As previously discussed, the greatest energy burden of growth for body size occurs in the first 3 months, the same time that body fat is being deposited at maximal rates. The human infant is therefore under strong selective pressures to meet the urgent energetic and nutritional needs during these first months of postnatal development. The following sub-section illustrates how the development of the infant immune system only further exacerbates this early energetic challenge.

Early immunity and growth impairment

Human infants are born with largely naïve adaptive immune systems that render them vulnerable to infectious disease. Notably, 44% of child deaths under the age of five take place during the neonatal period (WHO 2015). Therefore, the first postnatal months are when infants must rapidly build immunocompetence, or the ability to mount an adequate immune response. An enormous amount of immune activity occurs in the first few months of life, including peak production of lymphocyte, memory cell, and interferons (McDade and Worthman 1999; Denny et al. 1992; Haines et al. 2009; Hannet et al. 1992; Pirenne et al. 1992). Thus, immunity is an active process that can suddenly require the divergence of limited resources during the first months away from growth, toward maintenance.

Pathogenic assaults can in fact exert enormous costs on growth. Populations with high disease burden can have average -1.0 to -1.5 z scores (standard deviations relative to standard growth curves) for height, and -2.0 to -2.5 z scores for weight, body mass index and head circumference (Prentice and Darboe 2008). Many infections are symptomatic (e.g. diarrhea, malaria, pneumonia, HIV) and the costs on growth are well understood, but others are subclinical (e.g. hepatitis B, cytomegalovirus, Epstein-Barr virus, herpes, Helicobacter pylori), making it difficult to diagnose and treat. Many young children with growth impairment in developing countries become infected by multiple pathogens which initiate a downward cycle of infection, suppressed appetite, malabsorption, reduced growth, lowered immunity, and then repeated infection (Prentice and Darboe 2008), generating enormous deficits in growth. The sensitivity of infant growth to infection and malnutrition is well documented, such that measurements of weight and height are widely used to assess health disparities among and within populations (Johnston 1998a; WHO 1995). Delayed or prohibited growth due to infection can indeed exert long-term effects on health, productivity and quality of life.

Given the complexity of early immune development and the paradoxical associations of early childhood exposures and illness with immunocompetence (Asher et al. 1995; Isolauri et al. 2010), much attention has focused on the assessment of body composition during infancy as a measure for evaluating acute health (Olhager et al. 2003) as well as predicting future health outcomes (Thompson 2012; Kuzawa et al. 2007). Measures of body composition can indicate growth based on the LHT premise that energy diverted to maintenance (immune burden) will be necessarily syphoned away from growth, particularly in the case of an aberrant microbiome development. Growth deficits can indirectly indicate disease burden, and infants at the lower and upper ends of growth curves are at risk for a range of acute and future health problems. While infants at the lower ends of the growth curve may reflect high immune burden, infants at the higher end can have obesogenic profiles that have been associated with higher risk of childhood and adult obesity and a host of metabolic disorders later in life.

Thus, this paper examines differential growth outcomes to explore relationships between the microbiome and health, and explores the underlying microbial correlates for these differential health and growth outcomes. The following sub-section defines growth and development, and then explores a growing body of literature describing the underlying reasons for growth deficits in the studied population, both social and microbial.

Defining growth and development

Human growth may be defined as a quantitative increase in size or mass (Bogin 2010), and the longitudinal measurements of height in centimeters or weight in kilograms indicate how much growth has taken place in an individual. Development can be defined as a progression of changes, either quantitative or qualitative, that lead from an undifferentiated or immature state to a highly organized, specialized, and mature state (Bogin 2010). Human growth and development have evolved, sometimes as discrete processes, but more often as an integrated series of biological events (ibid).

A growth reference is a table or chart summarizing how an anthropometric measurement such as height or weight changes over the studied period of time (Johnston 1998b). The World Health Organization's (WHO's) international reference standards of growth are internationally accepted standards that were drawn from the WHO Multi-centre Growth Reference Study (MGRS), which was undertaken between 1997 and 2003 in order to generate new growth curves for assessing the growth and development of infants and young children around the world (WHO MGRS 2016). The MGRS collected primary growth measurements and related survey data from approximately 8500 children from various ethnic and cultural backgrounds from Brazil, Ghana, India, Norway, Oman and the U.S., and are considered the best international description of physiological growth for all children from birth until 5 years of age (Ibid). These reference standards establish the breastfed infant as the normative model for growth and development (Ibid).

When defining growth for a population or an individual, either a Z-score or an elected extreme centile is used. These metrics help to identify children who are unusually small or large, transforming a measurement into a standardized, relative measure. The formula for Z-score is: Z= (Measurement- Mean)/Standard Deviation. Where measurement equals the mean of the distribution, and Standard Deviation equals the standard deviation of the distribution (Johnston 1998b). In studies of growth, a Z-score provides a standard indicator of either a group or individual that are different from the reference value. In other words, Z-scores provide a way of comparing deviations from the reference. In a normally distributed population, a particular Z-score indicates the proportion, or percentage of the distribution above and below that value. For example, the proportion of a population below -2 Z-scores is 2 percent, while the proportion below -1 Z-score is 16 percent (Johnston 1998b).

A low-length-for-age (Zlen) is referred to as **stunting** and assessed as a value for length that is greater than 2 standard deviations below the reference mean for age (ie, a Z score that is <-2) (Johnston 1998b). A low weight-for-length-for-age (Zwfl) is called **wasting** and is generally defined as a value that is less than -2SD of the reference value. Stunting may or may not be accompanied by wasting. In some instances, weight-for-age (Zwei) may be used as the criterion of failure to grow, in which cases a Z-score of <-2 is termed **underweight**.

In terms of etiology, stunting is usually interpreted as a **chronic condition** caused by the accumulative effects of mild-to-moderate nutritional deficiency and/or diarrheal disease. In the absence of wasting, stunting indicates an overall proportional faltering of growth as the effects of environmental factors accumulate through time (Johnston 1998b). Wasting on the other hand, is more likely to be an **acute condition**, where the body is depleted of its nutrient reserves. Weight loss can occur and height may or may not be affected, depending on the specifics of the condition, including its duration and severity. Stunting is widespread among the world's developing countries, although wasting less common. It is clear the type of growth failure, and by extension the ecological conditions responsible vary among the developing nations of the world (Johnston 1998b). In this chapter I will use growth standards in three distinct contexts: first to summarize the anthropometry of the study population as a whole, second to monitor the growth status of individual children, and finally to explore how different social-ecological factors may influence relationships between infant microbiome development and growth.

The social determinants of growth

Differences in human growth can also be viewed as an indicator of social inequality or differential access to nutrition and resources among populations and within subpopulations. Biocultural and longitudinal studies of growth over time in a particular setting help researchers determine social inequality, and/or changes in social systems that differentially affect growth. This research attempts to discover the main social and material pathways that most significantly influence the growth, including composite measures such as a family's socioeconomic standing, or specific factors like parental education, family size, subsistence strategies and access to foods and other effects of urbanization. For most societies, these factors are inter-correlated and teasing out the factors that affect growth and development are challenging, and studies accounting for the social/material factors that influence the growth process, as well as the biological elements of weight gain, and body composition during early life in humans are key to elaborating our understandings of these biocultural phenomena. Section II assesses the broader social, economic, environmental and microbial factors that directly or indirectly influence growth outcomes in this Salvadoran study population to better illuminate these complex, interconnected processes.

Microbial correlates of growth

Emerging evidence suggests that development of the intestinal microbiome is a critical pathway linking early environments to later metabolic conditions (Edwards and Parrett 2002). The first evidence that gut microbes play a role in adult obesity came from studies comparing intestinal bacteria in obese and lean adults. Turnbaugh et al. (2009) found that lean individuals tended to have a more diverse microbiome—particularly with a higher proportion of Bacteroidetes that specialize in breaking down otherwise indigestible plant fibers that help maintain intestinal health and microbial diversity. Similarly, obese adults have more Firmicutes than lean controls (Turnbaugh and Gordon 2009), a phylum that specializes in increased fatty acid absorption and energy harvest, and is associated with obesity in animals and humans (Turnbaugh et al. 2009). Moreover proportion of Bacteroidetes increased with weight loss (Ruth E. Ley et al. 2006b), suggesting that bacterial type may be mechanistically related to body weight regulation.

Causality between bacteria and weight changes was supported by an elegant series of germ-free rodent studies conducted at the Gordon Lab. Researchers found that mice with microbiota from an obese twin gained more weight when compared with genetically identical mice receiving gut microbiota from the lean twin, despite identical diets (Ridaura et al. 2013). Moreover, co-housing the two groups of mice instigated a transformation in the mice with previously obese phenotypes, and prevented the development of increased adiposity and body mass (ibid). However, this reversal was blocked when researchers began feeding the mice a specially prepared unhealthy chow that was high in fat and low in fiber (as opposed to the usual high-fiber, low-fat mouse kibble) (ibid). Given this “Western diet,” the mice with obese-type microbes proceeded to grow fat even when housed with lean cage-mates. The unhealthy diet somehow prevented the bacteria associated with a lean profile from taking over. These early studies of gut microbes and adult obesity suggested that weight gain was in part a result of a combination of factors: the increased production of short chain fatty acids (SCFAs) (Guarner and Malagelada 2003), increased energy extracting capabilities (Cani and Delzenne 2007; Turnbaugh et al. 2006), as well as efficient energy absorption and adipose development (Nass et al. 2010).

Growing evidence suggests that the composition of the microbiome similarly may be associated with weight gain and adipose deposition during infancy. One prospective study of Belgian infants suggests early microbial differences precede the development of obesity. Vael et al. (2011) found that BMI z-scores between 1 and 3 years old were positively associated with levels of *Bacteroides* at 3 and 52 weeks of age and negatively with *Staphylococcus* at 3 and 26 weeks of age, controlling for infant feeding and several other risk factors for higher BMI. Another longitudinal study of infant feeding, microbiota development, and infant growth over the first 15 months of life in mainly breast-fed infants identified phyla-level changes in bacterial colonization with feeding transitions (Thompson 2012). The proportion of Firmicutes-type bacteria increased with both the cessation of breastfeeding and the introduction of formula, and higher proportions of Firmicutes were associated with subsequently greater weight gain and higher adiposity, potentially linking changes in early feeding to later obesity risk (ibid).

Recent experimental studies have contributed to understandings of how the early development of the microbiome may be related to obesogenic phenotypes in later life. Cho et al. (2012) showed that early-life sub-therapeutic antibiotic treatment increased fat mass, and altered metabolic hormones, hepatic metabolism, and microbiota composition. Building from this model, Cox et al. (2014) similarly administered low-doses of antibiotics to mice to examine whether timing of exposure (community disruption) is critical. Indeed, perturbations in the early months of microbial development (pre-weaning) had long-term effects on host metabolism. Moreover, even when exposures were limited to infancy and microbiota returned to normal population structure, the elevated adiposity emerged in early adulthood. Lasting metabolic consequences provide evidence for an early life “critical window” of host-microbe metabolic interaction, which would set up metabolic profiles for the remainder of the life course (Cox et al. 2014). Finally, they found that specific organisms were consistently under-represented in the mice that received antibiotics prior to metabolic phenotype development, which lends weight to the idea that the loss of key microbes during a critical window can be detrimental to long-term microbial diversity. These findings are consistent with prior studies showing that early life changes in metabolism or exposure to antibiotics predict later adiposity in rodents (Knittle and Hirsch, 1968) and humans (Ajslev et al., 2011; Murphy et al., 2013; Trasande et al., 2013).

The microbial correlates of undernutrition in infants and children has recently claimed the attention of researchers who are interested in clarifying associations between intestinal microbes and early growth mechanisms. A longitudinal study of the infant microbiome in Bangladesh showed that infants with moderate levels of acute malnutrition have “immature” gut microbiota when compared to healthy infants, and children with the most severe acute malnutrition had the most delayed microbial development (Subramanian et al. 2014). Treatment of severely malnourished children with therapeutic foods produced only incomplete and transient improvement in this immaturity (ibid), suggesting that the microbiome itself may be part of this larger picture.

Building upon this study, Blanton et al. (2016) also showed that perturbations in the normal development of gut microbiota were related to undernutrition in Malawian infants, and then tested these relationships in mice. Similar to the earlier rodent studies on adult obesity, microbiota from malnourished and healthy infants were transferred to germ-free mice. Unsurprisingly, the mice colonized with microbiota from healthy donors gained significantly more weight and lean body mass than mice colonized with microbiota from undernourished donors, despite identical diets (ibid). Interestingly, recipients of microbiota from healthy or undernourished 6-month-old donors grew more than recipients of microbiota from 18-month-old healthy or undernourished donors. Distinct microbial profiles were found at these two ages and various species were significantly correlated with weight gain and/or lean mass gain. These findings suggest that successional microbiota development is optimized to satisfy the different growth needs of the infant at different stages of development.

The mechanisms by which gut microbes initiate and support the metabolism are increasingly being identified and defined. An obvious mechanism identified early on is the enhanced caloric harvesting of carbohydrates and the effects on energy balance and weight gain (Turnbaugh et al. 2006). Up to 15% of carbohydrates are indigestible to humans, but are instead fermented by bacteria in the large intestine. These carbohydrates provide energy to support microbial metabolism, while producing short chain fatty acids (SCFAs) that are then absorbed and metabolized as an energy source by the host colonocytes (den Besten et al. 2013). Metagenomic screening has revealed that SCFA synthesis genes are commonly expressed by the GI microbiota, suggesting carbohydrate fermentation is a main function (Qin et al. 2010). Although SCFAs have numerous independent effects on weight gain, they have been shown to stimulate hepatic triglyceride synthesis and adipogenesis in white adipose tissue (Bäckhed et al. 2004; Xiong et al. 2004).

Just as the GI tract is home to the gut microbiome, it is also a locus of hormone production. This is particularly relevant to the microbial correlates of infant metabolism, as hormones help to negotiate and regulate the short and long term balance of resource allocation among growth and maintenance (Worthman 1999). During early developmental windows, endocrine regulatory channels can be adjusted in order to affect long-term life history priorities (ibid) including metabolic profiles (Kuzawa et al. 2007). Hormones modulate the metabolism by regulating the pace of growth and the timing of developmental transitions (Worthman 1999). It therefore is likely that gut microbes interact with and produce hormones involved in energy homeostasis (such as insulin, glucagon, leptin and ghrelin) and/or growth (for example, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) (Hansotia and Drucker 2005). Alterations in the populations of the GI microbiota may change the intra-community metabolic interactions (Gesta et al. 2007), and can globally affect host metabolic, hormonal and immune homeostasis (Reikvam et al. 2011).

Until recently, the guiding theory of infant development presumed that postnatal organ and systemic growth in mammals was mediated by the activity of the somatotropic axis, in which growth hormone (GH) (produced by the pituitary gland at the base of the brain), controlled growth through a circulating insulin-like growth factor–1 (IGF-1) (Butler and Le Roith 2001). This traditionally accepted theory has since been modified by the discovery that many tissues express IGF-1, and that the hormone can act independently of GH production (Lupu et al. 2001; Butler and Le Roith 2001; Efstratiadis 1998; Kaplan and Cohen 2007), although the mechanisms involved in the activation of IGF-1 have been unclear.

Schwarzer et al. (2016) recently found that mice with “normal” microbiota and germ-free mice had similar levels of GH, but the latter group had decreased activity of IGF-1 in their blood, liver, and muscles, and do not develop as much muscle or bone growth. Although the exact contribution of gut microbiota to hormone production, activation, and thus postnatal growth is unclear, injecting germ-free mice with IGF-1 brought up their growth on par with the typically colonized mice. Moreover, seeding germ-free mice with a single strain of lactobacillus had a similar outcome, suggesting that gut microbiota promoted growth by facilitating IGF-1 production and activity (Schwarzer et al. 2016). Other researchers also have found growth-promoting taxa (Blanton et al. 2016), although the exact mechanisms by which these gut microbes communicate metabolically with other tissues remain to be defined.

Gut microbiota involvement during times of undernutrition also have been tied to the activities of the somatotropic axis. A current explanation for the complex mechanisms underlying growth impairment due to under-nutrition involves GH resistance (Thissen 1994; Fazeli and Klibanski 2014). It has been suggested that tissue resistance to GH causes a drop in the production of IGF-1, leading to a delayed development and reduced size of an individual compared with age (Hizli et al. 2007). A key element in this relationship is likely IGF-1, whose production and activity are in part facilitated by microbiota (Schwarzer et al. 2016). Germ-free infant mice enter a GH-resistant state upon chronic undernutrition, and healthy microbiota is necessary and sufficient to boost postnatal growth by enhancing GH sensitivity and thereby increasing IGF-1 activity in peripheral tissues. In addition, Schwarzer et al. (2016) demonstrated that a selected strain of *L. plantarum* can recapitulate the beneficial effects of the microbiota on the somatotropic axis and on mouse juvenile growth, a functionality that may be shared with other commensal bacteria in the microbiota. Thus a disruption of the microbiota, as in the case of malnutrition, may be a key link between microbes and impaired growth (Smith et al. 2013). In contrast to healthy microbiome development, an immature microbiota appears to cause a form of delayed growth when nutrients are limited (Blanton et al. 2016).

More recently, specific microbes have been investigated for their ability to regulate appetite, affecting the production and storage of adipose tissue. *Helicobacter pylori,* for example, decreases appetite by modulating levels of the hormone ghrelin (Tatsuguchi et al. 2004). Ghrelin is a recently discovered GH-releasing peptide that may influence acute and chronic energy balance by increasing appetite and GH secretion, thereby increasing body weight and promoting adipogenesis (Nass et al. 2010; Castañeda et al. 2010). Ghrelin is upregulated during periods of rapid growth and is positively associated with weight and length gain in the first months of life (James et al. 2004). This upregulation may promote increased appetite and greater energy intake facilitating adipose tissue deposition during early infancy. *H. pylori* was once abundant in the human digestive tract but is now rare, due to more hygienic living conditions and the use of antibiotics (Blaser 2014). One of the unintended counter-effects of *Helicobacter pylori* eradication therapy is subsequent obesity (Tatsuguchi et al. 2004).

This small but growing body of research is beginning to explore the microbial correlates of metabolic conditioning during the early months of life. Taken together, the results indicate that human-associated gut microbiota are important for human growth and development, and are also a key pathway linking early environments with social and ecological variables to later metabolic and health outcomes (Edwards and Parrett, 2002).

As previously discussed, the first year of life is a period of rapid growth and development, somatically, neurologically, immunologically and microbially. During this time, infant body weight triples, brain size doubles, and the percentage of body fat peaks by 5-6 months (at 200%) (Kuzawa 1998; Bogin 2010; Holland et al. 2014). The greatest energetic burden of growth for body size occurs in the first 3 months of life, while at the same time body fat is being deposited at maximal rates. As infant body and brain size increase, maintenance costs increase, and yet body fat is relatively inexpensive to maintain. Therefore, body fat is laid down while maintenance costs are low and the infant is smaller, although costs remain high for body and brain growth. At the same time, the immune system is proliferating and building competence. Infants in these early months are particularly vulnerable to infectious disease, and growth lost in these early months due to infection can be difficult to recoup. This creates a high premium on early energetic deposition as a buffer against growth loss.

This suite of changing energetic requirements produces strong selection pressure for energy availability and usage: infant diet extraction ability and metabolic efficiency should be selectively favored to gain the most possible from available nutrients (Sheets and Worthman 2014). This is particularly true, in light of the limited milk diet of the first six months, requiring a specialized extraction efficiency (Hinde and Lewis 2015; O'Sullivan et al. 2013).

The developing microbiome and its infant host are under strong and to a degree shared selective pressures to meet the urgent energetic, nutritional, and immunologic needs during the early postnatal months. A growing microbiome can enhance energy extraction and fat deposition, helping to create more energy for both growth, fat deposition and maintenance. When the infant falls ill, fat stores can help buffer the brain and ameliorate infant somatic growth loss from illness or undernutrition. Moreover, a healthy microbiome can help during catchup growth to restore the lost growth during the acute infection. Dysbiotic or unbalanced microbiota seem to be part of a negative feedback loop during chronic infection, however. These dynamics underline the importance of understanding the interconnected relationships between the developing microbiome and host growth phenotypes.

Research questions and hypotheses

This chapter is based on the premise that the infant gut microbiome affects host metabolism and fat deposition, thus shaping growth curves during infancy.

Furthermore, that growth outcomes can in turn elaborate the story of a developing microbial community. In order to explore these multi-directional pathways of growth and development, I investigate the interactions between microbes and weight gain, as well as between social variables and weight gain more generally.

Q1: Are infant microbiota associated with different growth outcomes in this Salvadoran population?

> **Q1H1**: Bacteria that are associated with milk degradation such as *Lactobacillus*, *Veillonella*, *Bifidobacteria*, *Bacteroides, Megasphaera,* and *Ruminococcus* should positively correlate with growth outcomes in this population due to their milk fermentation abilities, and pathogenic or pathobiont bacteria (i.e. *Staphylococcus*, *Corynebacteria* and *Clostridia*) should negatively correlate with growth measures.
>
> **Q1H2**: Distinct bacterial profiles should be associated with different infant ages and stages of development.
>
> **Q1H3**: Infants of different growth phenotypes should have distinct profiles associated with their growth outcomes. Infants of the underweight growth phenotype (UGP) and overweight growth phenotype (OGP) groups should have less stable microbial profiles (indicating inflammation and/or dysbiosis), while infants from the average growth phenotype (AGP) group should have the “healthiest” microbial development with optimized microbiota for milk, followed by carbohydrate fermentation and assymilation.

Q2: What behavioral, health or environmental factors influence growth outcomes in this population?

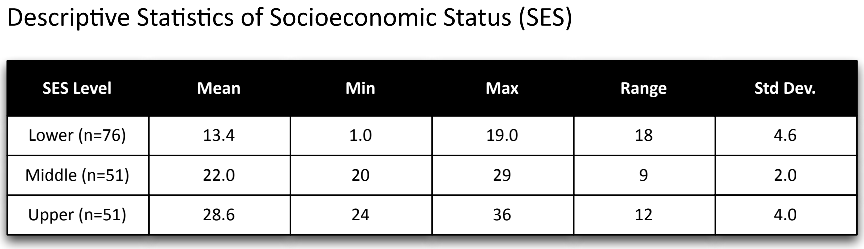
> **Q2H1**: Indicators of disease burden (i.e. total counts of illness episodes and medical interventions) should negatively affect growth outcomes in this population.
>
> **Q2H2**: Known social and economic factors that moderate growth (maternal education, SES and early feeding practices), and untested measures of household constitution (i.e. Household Microbial Ecology Index, or HMEI) should postitively influence early growth outcomes in this population. In the case of early feeding, I predict that breastfed infants grow slower and ultimately are smaller than their formula fed cohort.
>
> **Q2H3**: Formula fed and mixed-fed infants should grow more, be heavier, and yet should have higher rates of illness than breastfed infants.

Q2H4: Increased antibiotic usage should lead to greater infant growth/weight gain.

Section II: Case study of a Salvadoran Infant Population

Study population and setting

Joya de Cerén is a small semi-rural community located approximately 36 kilometers from the capital city of San Salvador, El Salvador. Located in a lowland valley, this region has a tropical climate with pronounced wet and dry seasons. Over 85% of the population identify as "mestizo" or mixed Indigenous Native American and European Spanish origin, another 12% identify as mostly European descent, while the remaining 1% identify as purely Indigenous descent (*Pipil* or *Lenca*). The community was initially founded in the 1950s as an agricultural reform project and additions to the development have been made since, in the way of dispersed settlements, currently totaling 6313 inhabitants. This population has largely



Descriptive Statistics of Socioeconomic Status (SES)

SES Level	Mean	Min	Max	Range	Std Dev.
Lower (n=76)	13.4	1.0	19.0	18	4.6
Middle (n=51)	22.0	20	29	9	2.0
Upper (n=51)	28.6	24	36	12	4.0

Table 25: Descriptive Statistics of Socioeconomic Status (SES).

summary statistics for each SES level. SES ranged from 0-36 on the scale and included metrics such as education, maternal work status, household status, monthly household income, and family remittance.

transitioned from subsistence-based agriculture to a struggling market economy. Households are poor, with an average of 6 people living in each dwelling. Unemployment rates are high, and on average only 1-2 adults financially support the entire household.

Descriptive Data of the Study Population, n = 71

Variable	Subgroup	Mean	Median	Iq Range or %
Mother's Age (yrs.)		25.3	24	(21-30)
Mother's Education		8.5	9	(6-12)
Gender	Male	38		54%
	Female	33		46%
Infant Age at Baseline (mo.)		4.8	4	(1-7)
Infant Age at 2nd Collection (mo.)		8.2	7	(5-11)
Infant Age at 3rd Collection (mo.)		12.8	12.5	(9-16)
Birth Mode	Vaginal	53		75%
	C-Section	18		25%
Birth Weight (gr)		3049.8	3005	(2797-3473)
Birth Length (cm)		48.4	49	(47-50)
Days of hospitalization		2.7	0	(0-3)
Antibiotic Administration		2.3	2	(1-3)
Diarrhea Episodes		2.8	2	(1-4)
Upper respiratory		1.8	1	(1-3)
GI		1.2	1	(0-2)
Fever		0.5	0	(0-1)
Allergy		0.2	0	0
Total Illness		3.7		
Early Feeding	Pure Breast milk (BM) (n=16)			23%
	Predominantly BM (n=28)			39%
	Mixture (n=9)			13%
	Predominantly Formula (n=8)			11%
	Pure Formula (n=10)			14%
Currently Breast Feeding	Yes (n=154 obs)			
	No (n=28 obs)			
Birth Complication	Yes (n=37)			
	No (n=34)			
Birth Timing	Premature (n=4)			
	Term (n=64)			
	Post-mature (n=4)			
HMEI Scale	Lower (n=10)			
	Middle (n=25)			
	Upper (n=23)			
SES Levels	Lower (n=28)	13.64	14	7.5
	Middle (n=19)	22.32	22	3
	Upper (n=21)	28.5	28	5

Table 26: Descriptive statistics of the study population.

Study participants included 71 infant and primary caregiver pairs. Participants were recruited through the local public health worker. Infant participants were between the ages of 0 and 12 months at baseline (average age at baseline = 4.8 mo). Approximately one-quarter of the infants were cesarean section (see Table 26). This study population follows fairly standard weight and length growth curves (see Figure 41, Figure 40, Figure 41, and Figure 43), compared to

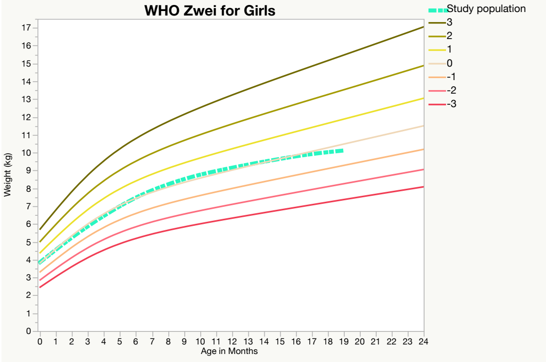


Figure 40: Female infants compared to WHO standards for growth (Z-weight scores).

The female infant study population is perfectly in line with 5oth percentile Zwei.

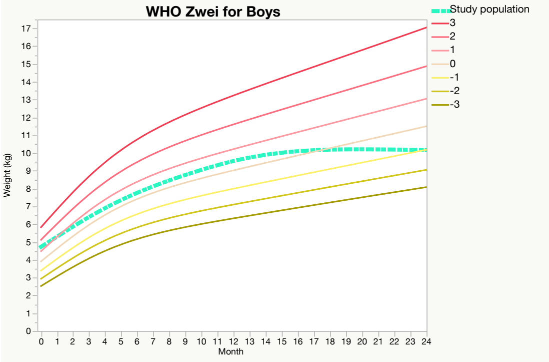


Figure 41: Male infants compared to WHO standards for growth (Z-weight scores).

Males in this population have slightly above the 50 percentile until around the 18th month.

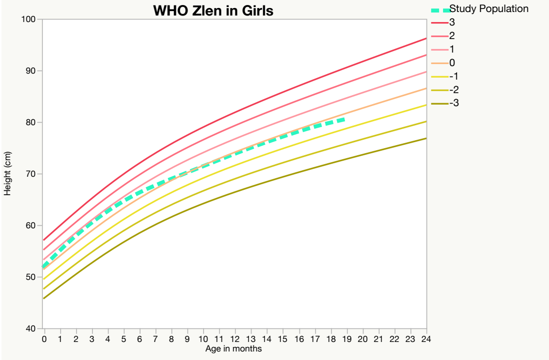


Figure 42: Female infants compared to WHO standards for growth (Z-length).

The female infants study population is at the 50th percentile Zlen

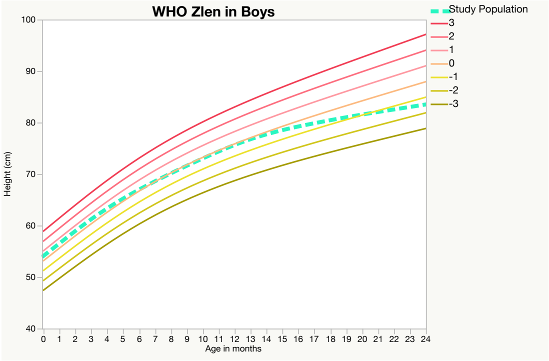


Figure 43: Male infants compared to WHO standards for growth (Z-length).

The male infant study population is in line with the 50th percentile Zlen, until around the 16th month.

WHO growth standards. Girls weight for age and length for age scores are standard, however the boys growth charts falter slightly in the middle of the second year. This is likely due to a smaller sample size in this later age and an outlier pulling the mean.

Methods

Over the course of one year, a minimum of three home visits and interviews with participating caregivers were conducted, during which anthropometric measurements (length and weight), and fecal samples were collected. At least three fecal samples were collected per infant, with additional sampling around key events (e.g. administration of antibiotics, severe illness, etc.). Disposable diapers were delivered to caregivers the night before a sample was collected, and soiled diapers were collected within two hours of infant defecation. Samples were collected with sterile swabs and stored at -20° Celsius for up to three months before being packaged on ice and hand-delivered to the Knight Laboratory at the University of Colorado at Boulder for sequencing.

Bacterial DNA was extracted from the samples using the Eppendorf EpMotion 5075 robot. Polymerase chain reaction (PCR) was then used to amplify the 16S ribosomal RNA (rRNA)[6] genes to characterize the taxa present in the gut

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[6] The 16S rRNA gene (often referred to as "16S") is widely used in studies of microbial ecology as a "barcode gene" (Hebert et al. 2003) to quantify microbial community structure and diversity (Pace 1997; Hugenholtz et al. 1998). The widespread use of 16S as a barcode gene has been driven in part by the fact that it is universal across bacteria and archaea, and can be easily amplified from a wide diversity of taxa at one time by the polymerase chain reaction (PCR). For these reasons the 16S gene is phylogenetically informative, because it can be used to identify and phylotype sequences based on extensive databases of 16S sequences with associated taxonomic and phylogenetic information (Pace 1997; Woese and Fox 1977). There are numerous advantages to using 16S as a microbial community barcode gene, but also numerous disadvantages including amplification and sequencing bias and error (Suzuki and Giovannoni 1996; Hong et al. 2009), difficulty with the accurate taxonomic identification and binning of short sequences (Qin et al. 2010; Kunin et al. 2010; Haas et al. 2011; Rodrigue et al. 2010).

Descriptive statistics on growth percentiles

Age Group	Length < 5%	Weight < 5%	ZBMI < 5%	Length > 95%	Weight > 95%	ZBMI > 95%
0-3 Mo.	3	2	2	1	2	5
4-7 Mo.	14	9	6	4	2	4
8-11 Mo.	4	5	3	1	3	2
12+ Mo.	8	7	7	1	3	4

Table 27: Descriptive statistics on growth percentiles.

This chart shows the number of infants in each group based on type of Z-score and age category.

Descriptive statistics on growth Z-scores

Age Group	Length < -2	Weight < -2	ZBMI < -2	Length > 2	Weight > 2	ZBMI > 2
0-3 Mo.	3	2	2	1	1	2
4-7 Mo.	9	7	4	1	0	2
8-11 Mo.	3	3	1	1	1	1
12+ Mo.	4	4	7	1	1	1

Table 28: Descriptive statistics growth groups (Z-scores).

This chart represents the number of infants that fall into each Z-score category per age group.

Descriptive statistics on Z-scores

Z-Score	Mean	Std Dev	Min	Max	Median
Z for length (zlen)	-0.31	1.29	-3.88	2.69	-0.20
Z for weight (zwei)	-0.24	1.20	-3.74	2.49	-0.17
Z weight for length (zwfl)	-0.04	1.22	-3.37	4.39	-0.16
Z for bmi (zbmi)	-0.07	1.22	-3.36	3.38	-0.17

Table 29: Descriptive statistics on the different Z-score measures.

Mean growth Z-scores

Z-Score	Z-Weight	Z-Length	Z-BMI
Z < -2	-3.312	-2.605	-3.339
-2 < Z < 2	-0.074	-0.091	0.034
Z > 2	2.307	2.413	2.703

Table 30: Mean growth Z-scores.

These are average Z-scores among the three growth phenotype groups.

microbial communities of these infants (n = 215 samples successfully analyzed). Samples were then sequenced on the Illumina second generation sequencer.

Alpha diversity[7] (α-diversity) was calculated using Chao1 diversity index based on the rarefied OTU table at a depth of 1226 sequences per sample. The calculations were performed either with the QIIME pipeline (Caporaso et al., 2010), with R software (R Foundation for Statistical Computing, Vienna, Austria) using the vegan (Oksanen et al., 2011) and picante (Kembel et al., 2010) packages, and/or JMP Pro 12 software. To compare between-sample variations in the composition of the total microbial community, unweighted and weighted UniFrac distances (Lozupone and Knight, 2005) were calculated with the QIIME pipeline (Caporaso et al., 2010). PCoA (Principal Coordinate Analysis) was performed on both distance matrices and coordinates were used to draw 3D graphical outputs.

The current study utilizes the World Health Organization's (WHO's) international reference standards of growth. All Z-scores (Z-weight, Z-length, Z-weight-for-length, Z-BMI) were normally distributed in this population. In this study, under-weight growth phenotypes (UGP) infants were defined as either a low length, weight or BMI for age ($Z<-2$, $P< 5\%$). Average growth phenotypes (AGP) were defined as infants who were between the two extremes ($-2 <Z< 2$, or $5\% <P< 95\%$). Over-weight growth phenotypes (OGP) were defined as infants who were in the highest ends of the growth curves ($Z> 2$, $P> 95\%$). Because the proportion of a population below -2 Z-scores is 2 percent, the Z-score measure was a more exclusive measure and was used for specific purposes during analysis.

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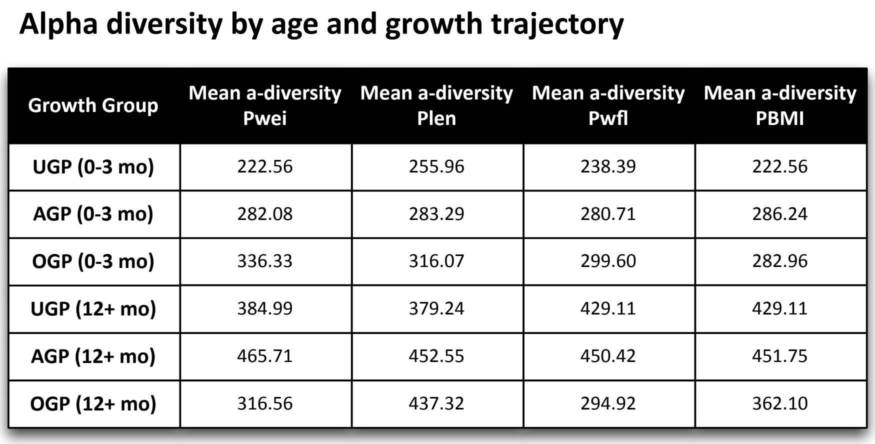
[7] Alpha diversity is a summary measure of intra-sample taxonomic diversity, which captures both the organismal richness of the sample and the evenness of the organisms' abundance distribution.

In certain circumstances the 5th percentile of growth was used to have a large enough sample size, as it screens in a larger proportion of children. In general however, the majority of analyses were conducted using the more extreme Z-score so as to avoid a high false positive rate. Infant overweight was defined as having a BMI above the 90th percentile and obesity was defined as a BMI above the 95th percentile.

For analysis, the age variable was used both as a continuous factor by month and a grouped-age variable (0-3 months, 4-7 months, 8-11 months, and 12+ months). These age groups were determined based on common life stages that occur around chronological transitions appropriate for this population. Infants 0-3 months largely received milk substrates, whereas mothers in this area tend to introduce “taste” complementary foods around the 4th month of life. Thus the 4-7 months group captured the transition to complementary feeding. The 8-11 months group is a transitional group where infants are often still receiving milk (either formula or breast), and 12+ months is commonly a time when infants will be weaned off of breast milk or bottle.



Alpha diversity by age and growth trajectory

Growth Group	Mean a-diversity Pwei	Mean a-diversity Plen	Mean a-diversity Pwfl	Mean a-diversity PBMI
UGP (0-3 mo)	222.56	255.96	238.39	222.56
AGP (0-3 mo)	282.08	283.29	280.71	286.24
OGP (0-3 mo)	336.33	316.07	299.60	282.96
UGP (12+ mo)	384.99	379.24	429.11	429.11
AGP (12+ mo)	465.71	452.55	450.42	451.75
OGP (12+ mo)	316.56	437.32	294.92	362.10

Table 31: A-diversity by age and growth phenotype group.

Among the 0-3 months age group, α-diversity tends to be lowest in underweight group phenotype (UPG) infants, higher in the average growth phenotype (AGP) infants, and highest in the overweight growth phenotype (OGP) infants. This trend changes at 12+ months, where AGP infants have the highest α-diversity when compared to the other two groups (See figure 6 & 7).

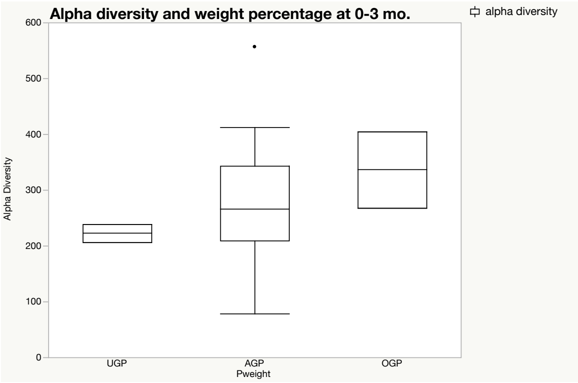


Figure 45: A-diversity and growth phenotype groups, ages 0-3 mo.

At 0-3 months, α-diversity tends to be lowest in underweight group phenotype (UPG) infants, higher in the average growth phenotype (AGP) infants, and highest in the overweight growth phenotype (OGP) infants. This suggests a certain "healthy range" for α-diversity in early infancy.

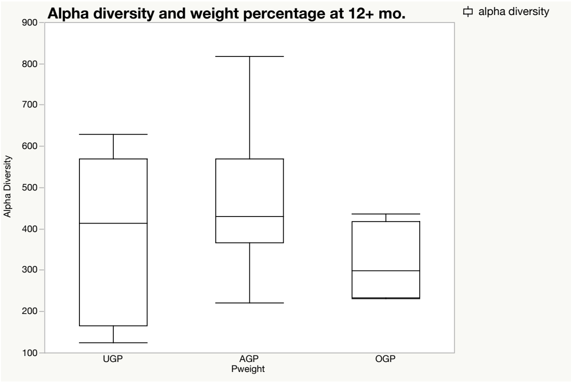


Figure 46: A-diversity and growth phenotype groups, ages 12+ mo.

At 12+ months, AGP infants have the highest α-diversity when compared to the other two groups. This suggests that as an infant microbiome reaches an adult-like stability and profile, higher α-diversity may be advantageous (if not in health outcomes, likely in weight gain and growth trajectories associated with these growth phenotypes).

Results

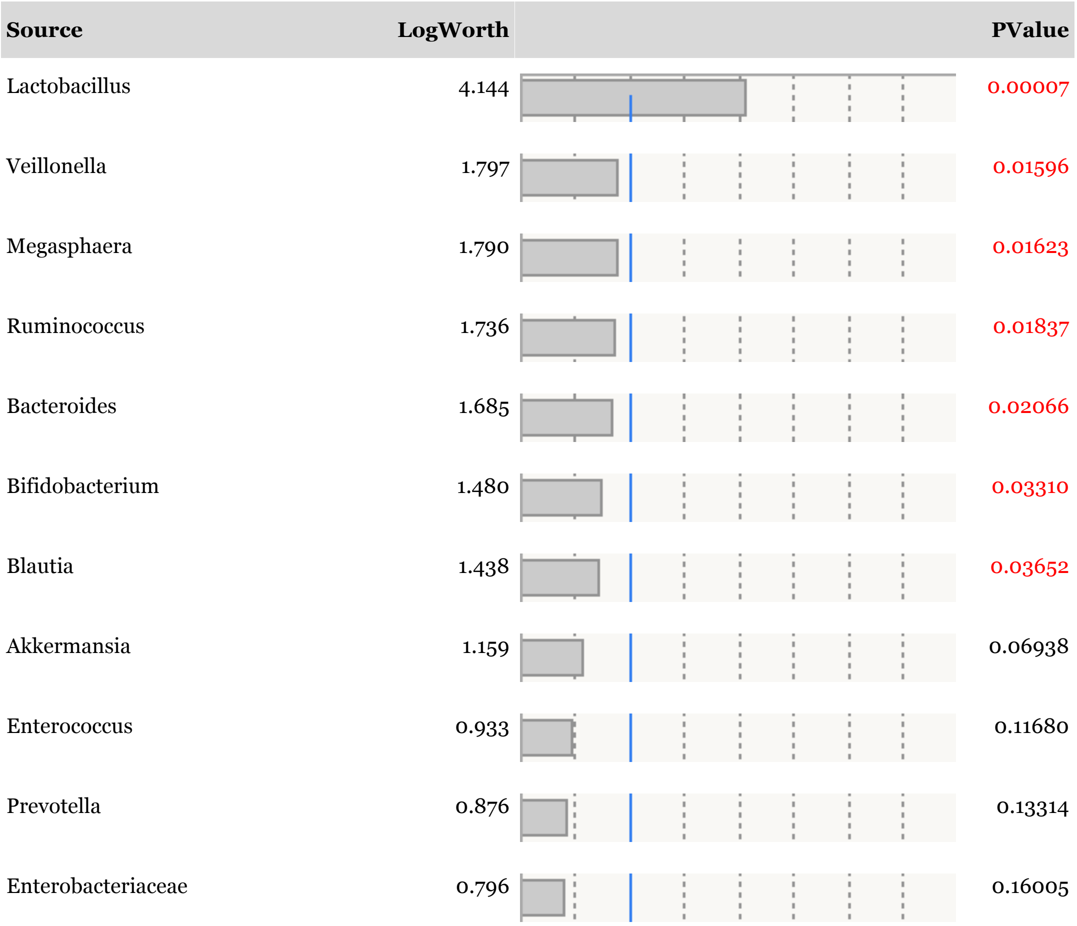
***Q1**: Are infant microbiota associated with different growth outcomes in this Salvadoran population?*

A-diversity is significantly related to multiple growth outcomes. In the aggregate population, overweight growth phenotype (OGP) infants had significantly lower α-diversity than did infants from the underweight growth phenotype (UGP) (P=.007), and from the average growth phenotype (AGP) (P=.047) when assessing the Pwfl growth measure. AGP infants also have significantly higher α-diversity than infants in the OGP group when examining PBMI (P=.032). Plen and Pwei were not significant measures.

Interestingly, early in life (0-3 mo), AGP infants have an α-diversity between that of the infants with UGP and OGP (see Figure 45 and Table 31). Once infants reach their first year of life, both extremes on the growth charts (UGP and OGP) have lower α-diversity when compared to the healthy growth range group (see Figure 46).

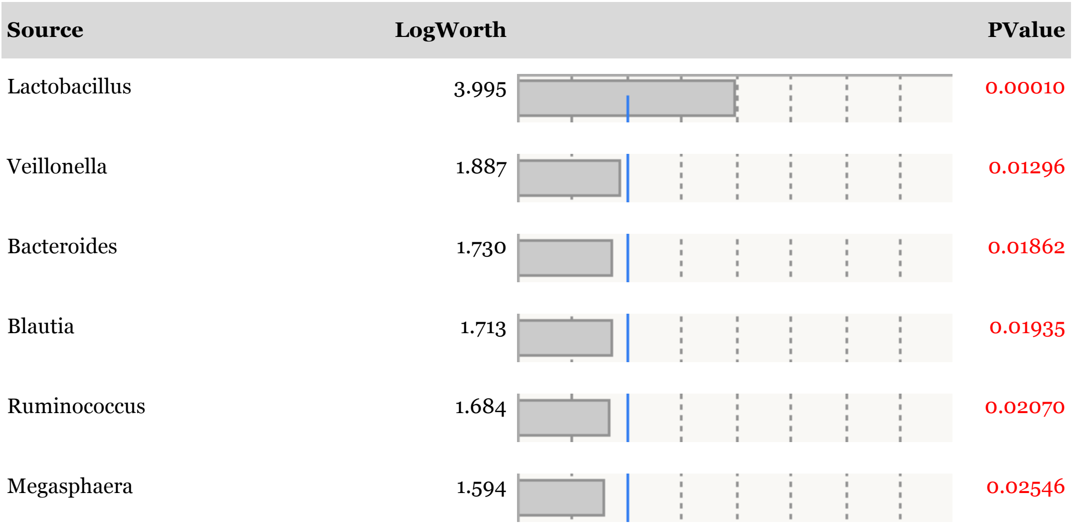
***Q1H1**: Bacteria that are associated with milk degradation such as Lactobacillus, Veillonella, Bifidobacteria, Bacteroides, Megasphaera, and Ruminococcus should positively correlate with growth outcomes in this population due to their milk fermentation abilities, and pathogenic or pathobiont bacteria (i.e. Staphylococcus, Corynebacteria and Clostridia) should negatively correlate with growth measures.*

To explore what microbes may be interacting with infant growth parameters, a standard multiple regression was performed to predict growth based on the relative abundance of specific bacterial genera. Preliminary analyses were performed to ensure there is no violation of the assumption of normality, linearity and multicollinearity. A significant regression equation was found (F= 2.22, P= .001), with an R^2 of .19 when investigating Zwei (Table 32). Zwfl was also found to have significant regression equation (F=2.5, P=.002), with an R^2 of .20 (Table 33). ZBMI had a significant regression equation (F=3.5, P=.0002), with an R^2 of .22. Zlen was only associated with *Lactobacillus* and *Veillonella* (F=1.8, P=.003, R^2=.12). These results indicate that *Lactobacillus, Veillonella, Megasphaera, Ruminococcus, Bacteroides, Bifidobacterium*, and *Blautia* are significantly related to infant growth during the first year of life. Thus, **Q1H1** was correct that bacterial genera associated with early milk degradation were positively correlated with infant growth in the first year +. However, the general premise of this chapter is that specific profiles of bacteria should have differential effects on growth outcomes, further investigation was required.

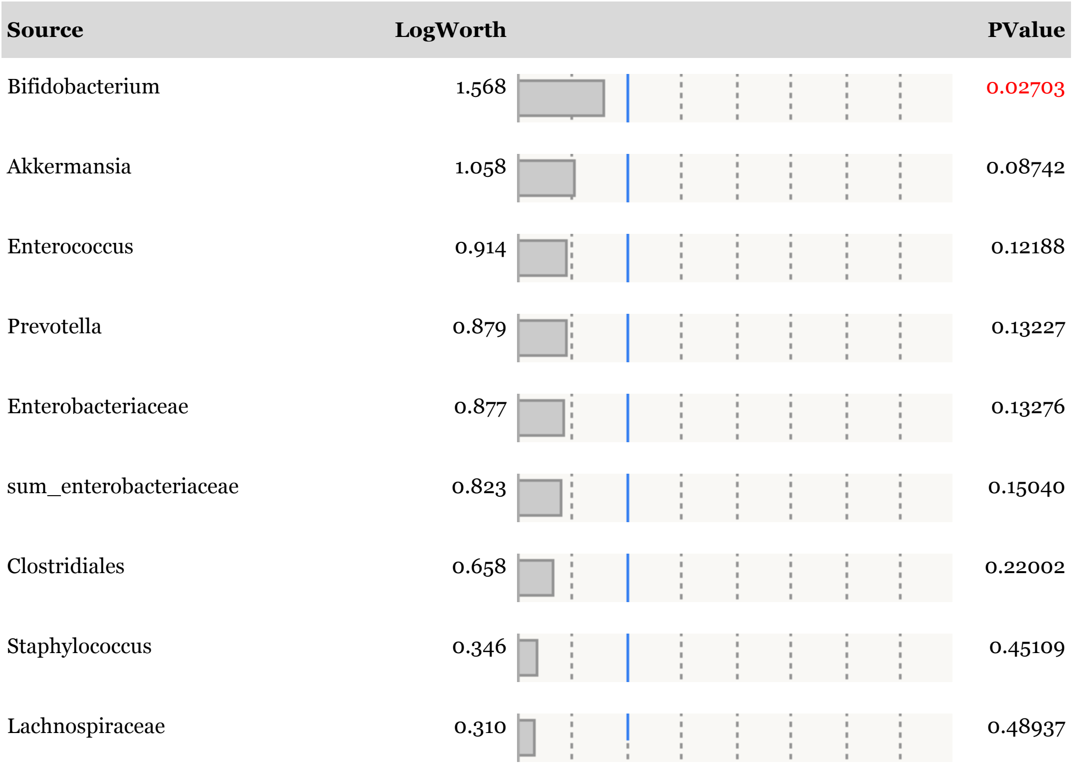


Source	LogWorth	PValue
Lactobacillus	4.144	0.00007
Veillonella	1.797	0.01596
Megasphaera	1.790	0.01623
Ruminococcus	1.736	0.01837
Bacteroides	1.685	0.02066
Bifidobacterium	1.480	0.03310
Blautia	1.438	0.03652
Akkermansia	1.159	0.06938
Enterococcus	0.933	0.11680
Prevotella	0.876	0.13314
Enterobacteriaceae	0.796	0.16005

Table 32: Bacterial genera ranked in order of significance to growth (Zwei).



Source	LogWorth	PValue
Lactobacillus	3.995	0.00010
Veillonella	1.887	0.01296
Bacteroides	1.730	0.01862
Blautia	1.713	0.01935
Ruminococcus	1.684	0.02070
Megasphaera	1.594	0.02546



Source	LogWorth	PValue
Bifidobacterium	1.568	0.02703
Akkermansia	1.058	0.08742
Enterococcus	0.914	0.12188
Prevotella	0.879	0.13227
Enterobacteriaceae	0.877	0.13276
sum_enterobacteriaceae	0.823	0.15040
Clostridiales	0.658	0.22002
Staphylococcus	0.346	0.45109
Lachnospiraceae	0.310	0.48937

Table 33: Bacterial genera ranked in order of significance to growth (Zwfl).

The key bacteria associated with Zwfl was the same bacteria associated with Zwei.

***Q1H2**: Distinct bacterial profiles should be associated with different infant ages and stages of development.*

Based on the understanding that the early development of gut microbiota is a non-random, sucessional process over time (Koenig et al. 2010), I hypothesized that different age groups would show distinct profiles of bacteria associated with growth. To test this hypothesis, the metabolically significant bacteria were determined at each age group, using the Zwfl growth measure.

At **0-3 months** *Sarcina*, *Clostridiales*, and *Bifidobacterium* were positively associated with Zwfl, with a regression equation (F=5.5, P=.001) with an R^2 = .47. At **4-7 months**, *Blautia* was positively associated with Zwfl (p=.01), while *Faecalibacterium* (p=.0001), *Corynebacterium* (p=.02), and *Dorea* (p=.0001) were negatively associated with Zwfl, with a regression equation (F=5.8, P=.0001), and R^2 = .23. At **8-11 months** *Megasphaera* (p=.003), *Enterococcus* (p=.009), *Enterobacteriaceae* (p=.02), *Ruminococcus* (p=.04)*, and Lactobacillus* (p=.05) were all positively correlated with Zwfl. The significant regression equation for Zwfl was (F=3.5, P=.004), with an R^2 of .48. At **12+ months**, *Akkermansia Enterobacteriaceae* (p=.008), *Prevotella* (p=.018)*, Bacteroides* (p=.02)*, Blautia* (p=.02), and

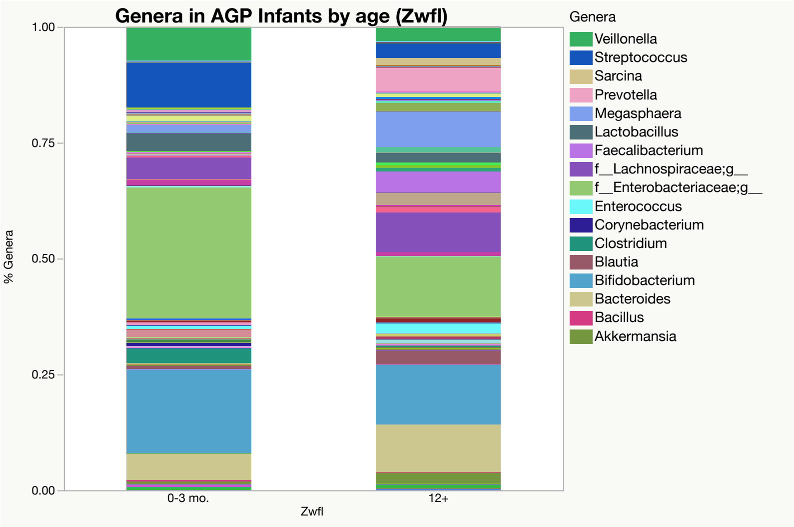


Figure 13: 100% stacked bar graph compares bacterial taxonomic composition among AGP infants at two different time points (0-3 mo. and 12+ mo.)

This highlights the changes in bacterial composition over time.

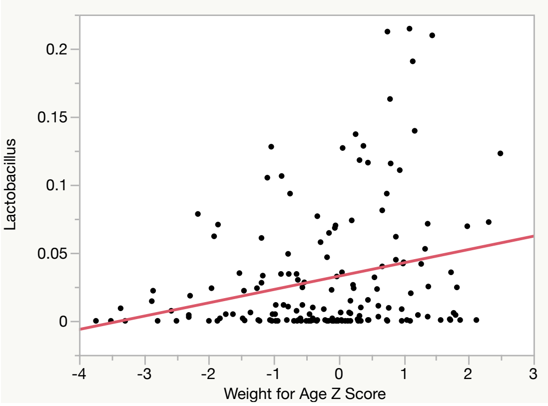


Figure 47: Lactobacillus and growth (Zwei).

Lactobacillus levels are positively correlated with weight (ZWei) scores.

Megasphaera (p=.035), were significantly and positively correlated with Zwfl, with a regression equation (F=3.1, P=.009), R^2= .46.

Thus, **Q1H2** was accurate in supposing that age groups showed different growth-related bacteria. In the first months, *Corynebacterium* was consistently negatively correlated with growth, which is unsurprising given its infamous toxin production and pathogenesis (Murphy 1996), and lending evidence to the later portion of ***Q1H1***. Also unsurprising was that many of the milk-degrading bacteria were positively associated with growth in the first months of life (i.e. *Bifidobacterium*, *Lactobacillus*, *Megasphaera,* and *Bacterioides*). It is also unsurprising that *Bacteroides*, a bacterium that can shift from milk fermentation to that of carbohydrates based on the infant's diet, would still be associated with growth in later months. However, to better understand the timing of key bacteria and their influence on growth, further investigation into growth phenotypes is necessary.

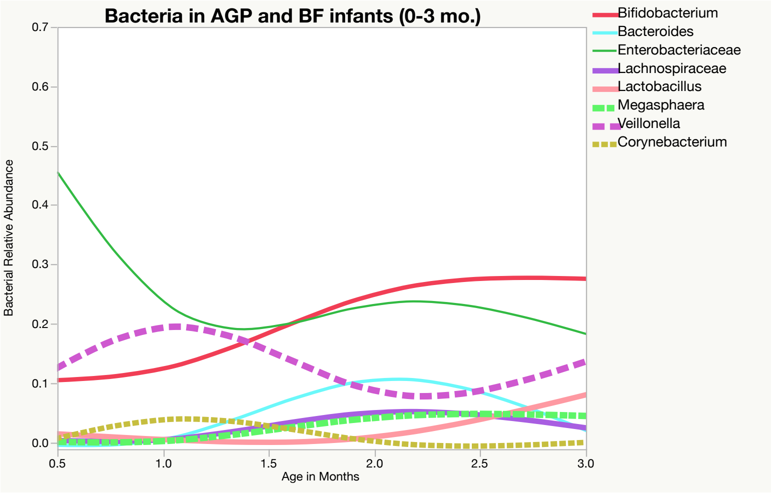


Figure 49: Bacterial progression over time among AGP infants, age 0-3 months.

Q1H3*: Infants of different growth phenotypes should have distinct profiles associated with their growth outcomes. Infants of the UGP and OGP groups should have less stable microbial profiles (indicating inflammation and/or dysbiosis), while infants from the AGP group should have the "healthiest" microbial development with optimized microbiota for milk, followed by carbohydrate fermentation and assymilation.*

Previously age-related changes in growth-associated bacteria were shown. To address the question if different microbiota effect changes in growth, analyses were conducted on the bacteria associated with different growth phenotypes, segregated by age (see Figure 53). First, infants of the average growth phenotype (AGP) were explored at two age points (0-3 months and 12+ months), using Zwfl as the measure of growth (see Figure 13). At 0-3 months, *Bifidobacterium* (P=.025), *Faecalibacterium* (P=.04) and *Ruminococcaceae* are positively correlated with Zwfl while *Blautia* (p=.035) is negatively correlated with Zwfl (see Figure 49 and Figure 52).

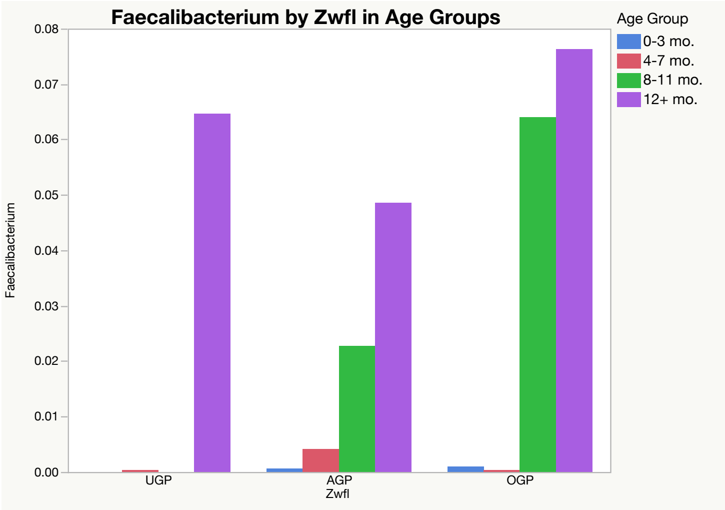


Figure 50: Faecalibacterium by Zwfl in age groups.

This bacterium becomes more prevalent in these groups in later months, and has the highest relative abundance in the OGP group.

At 12+ months, *Faecalibacterium* (P=.00056), *Corynebacterium* (P=.0029), *Ruminococcaceae* (P=.003), *Enterobacteriaceae* (P=.004), *Clostridiales* (P=.008), *Bacteriodes* (P=.012), and *Prevotella* (P=.032) are positively correlated with Zwfl and *Ruminococcus* (P=.015) is negatively correlated (R^2 = .98) (see Figure 51).

Thus, in AGP infants at 0-3 months, milk fermenting bacteria *Bifidobacterium and Ruminococcaceae* are positively associated with growth. At 12+ months these milk fermenters remain positively associated with growth, along with other complex carbohydrate fermenting bacteria including *Bacteroides, Faecalibacterium* and *Prevotella*. Although *Faecalibacterium* is well-known for its complex carbohydrate fermentation, it is often not commonly found in Malawian and Bangladeshi infants until after the first year of life (Blanton et al. 2016) and second year of life for Japanese infants (Koga et al. 2016). In this Salvadoran population, it not only began to proliferate early (by months 7) but it was found to be significantly associated with growth in AUG infants from the first months of life all the way through the second year. Furthermore, the OGP group has the highest levels of this bacterium (see Figure 50), suggesting that it may be a key player in weight gain and growth support for this population.

Under-weight growth phenotypes were evaluated using Pwfl. This was to ensure a sample size large enough to conduct the analyses (UPG = P < 50%). For infants at 0-3 months *Clostridiales* (P=.009) and *Enterococcus* (P=.037) are positively correlated with Pwfl, and *Erysipelotrichaceae* (P=.03) is inversely associated with Pwfl (R^2 =.58). For infants 12+ months

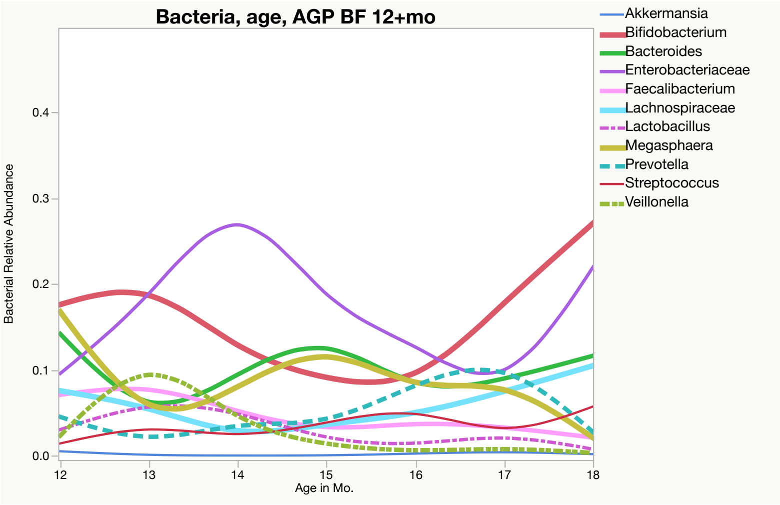


Figure 51: Bacterial colonization in AGP infants 12+ months.

Levels of different bacterial genera in infants of the average growth phenotype (AGP) group at 12+ months Bifidobacterium, Enterobacteriaceae, Bacteroides, Megasphaera are abundant in this population, while Lactobacillus is decreasing.

Enterobacteriaceae (P=.007), *Lactobacillus* (P=.028), and *Clostridiales* (P=.018) were positively correlated with Pwfl and *Streptococcus* (P=.0002) and *Ruminococcus* (P=.0083) were negatively associated with Pwfl (P=.006, R^2 =.85).

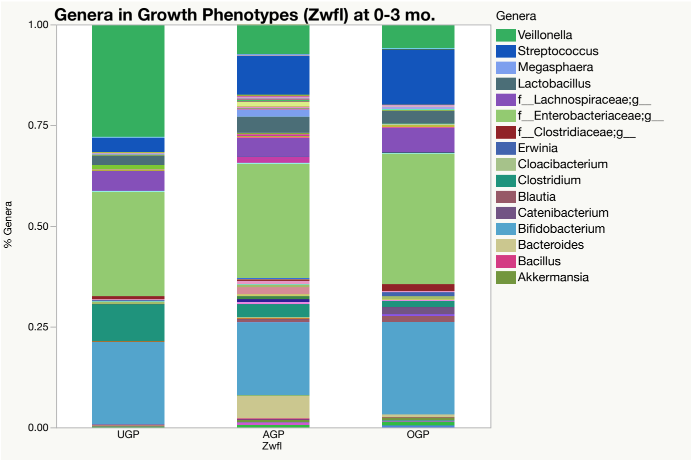


Figure 52: Bacterial abundances among growth phenotypes at 0-3 mo.

The early months of life show variation in bacterial composition among the growth phenotype groups.

For over-weight and growth phenotypes, Pwfl was also used (over-weight=P > 90%). In over-weight (OPG) infants 0-3 months, *Ruminococcus* (P= .03) was positively associated with Pwfl and *Clostridiales* (P=.018) was negatively correlated with Pwfl (P=.08, R^2=.44). For those 12+ months old OPG infants, *Megasphaera* (p=.029) was positively associated with Pwfl, while *Bacteroides* (p=.044), *Blautia* (P=.028), *Clostridiales* (P=.044) and *Lactobacillus* (P=.017) were found to be negatively associated with Pwfl (P=.047, R2= .98). While not found to be

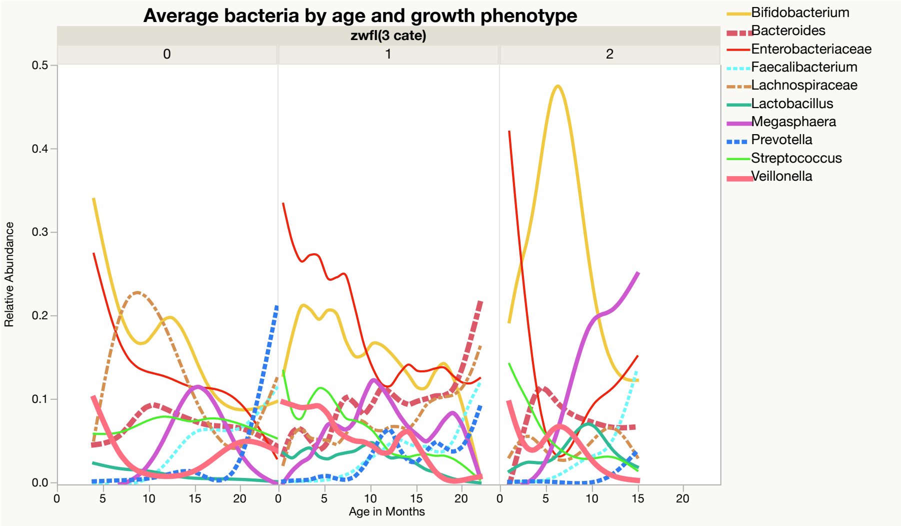


Figure 53: Average bacterial colonization among growth phenotype groups.

Growth category 0 = UPG infants, category 1 = APG infants, while category 2 = OPG infants. Bacterial profiles of the OPG group are less stable than the other two phenotypes, with high levels of Bifidobacteria. UPG infants show a delayed exposure to Megasphaera.

significantly related to weight gain in the OPG 12+ group, these infants had the highest relative levels of *Bifidobacterium* and *Faecalibacterium* compared to the other two growth phenotype groups, indicating high levels of SCFA production and energy absorption. Moreover, this OGP group had the highest relative abundance of *Megasphaera* which is found to be significantly related to growth in this age and GP category, at least in part explaining increased weight gain and growth.

Finally, the curiously low levels of *Lactobacillus* in this population demanded attention. *Lactobacillus* was not found in infants > 1 months, and many infants began to acquire this seemingly important microbe only in their second month. As previously mentioned, *Lactobacillus* was significantly correlated with Zwfl in the general population (P=.0001) (see Figure 47). It was also found in higher abundances in AGP infants, compared to UGP infants (P=.044, F=4.1). To explore the environmental and social variables that affect exposure to and the development of *Lactobacillus*, individual variables were analyzed. Early feeding practices were significantly correlated with the relative abundance of *Lactobacillus* (F=4.3, P=.015): BF and MF infants had significantly more *Lactobacillus* than FF infants (P=.01, P=.04 respectively). Some variables were surprisingly insignificant: birth weight, birth timing, and antibiotic exposure were found to be insignificant in

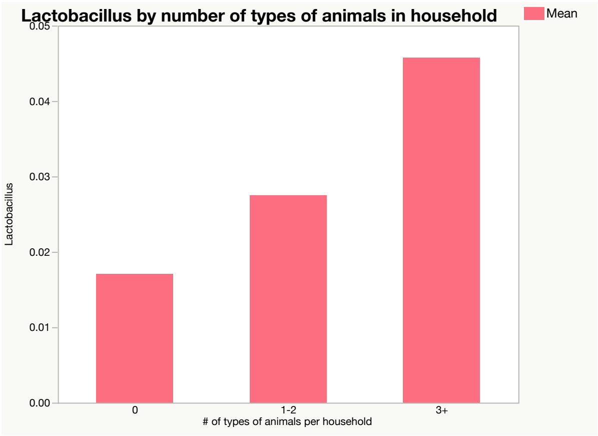


Figure 54: Number of animals in household predicts Lactobacillus.

The relative abundance of Lactobacillus increases as the number of types of animals in the household increase.

explaining levels of Lactobacillus. A surprising variable found to influence *Lactobacillus* levels was those who were exposed to more distinct types of animals had significantly more *Lactobacillus* (F=4.14, P=.017) (Figure 54). This was true at all ages > 2 months Interestingly, underweight (UPG) infants had the lowest levels of *Lactobacillus* at all age groups, and yet these bacteria was the most significantly related to growth among the group as a whole, suggesting that even at low levels this bacterium exerts strong influence over growth.

In conclusion, **Q1H3** is correct in that different growth phenotypes have different microbial profiles associated with growth. Moreover, OGP infants had the least stable microbial profiles (See Figure 48), although it may not be due to inflammation, but rather highly productive milk and carbohydrate fermentation. It also suggests that the UGP group may suffer a delayed and immature development of the microbiome, leading to inhibited growth outcomes.

Q2H1*: Indicators of disease burden (i.e. total counts of illness episodes and medical interventions) should negatively affect growth outcomes in this population.*

To test the **Q2H1** hypothesis that disease burden should negatively affect growth outcomes in this general population (measured by Zwfl), a standard multiple regression was performed. Preliminary analyses were first performed to ensure there is no violation of the assumption of normality, linearity and multicollinearity. A significant regression equation was found (F = 5.5, P= .0001), with an R^2 of .39. As to be expected, infants born underweight and those born prematurely had lower Z-scores (p=.0001, p=.045). Infants who had higher rates of diarrhea had significantly higher Zwfl scores (P=.003). Finally, infants who did not get sick had significantly higher Z-scores than those who had been sick 2 (P=.0018) and 4 (P=.0175) times. The surprising outcome here was that higher rates of diarrhea were associated positively with Zwfl scores, while total sum of sickness (including GI issues, upper respiratory illness, and fevers) was negatively associated with Z-scores.

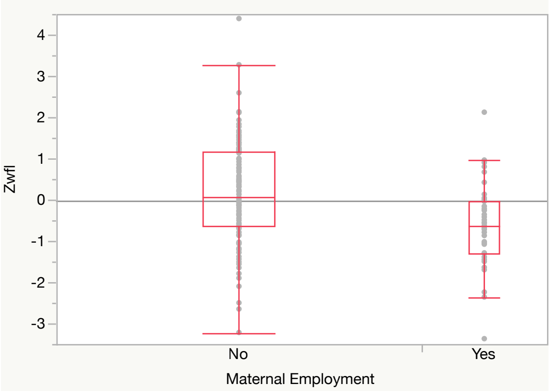


Figure 56: Maternal employment and Zwfl scores.

Infants with mothers who were formally or informally employed had lower Zwfl scores.

Next, infants who were below -1 Z-score were evaluated to see if indicators of disease burden would affect lower weight infants more strongly than the entire population. The Z-score -1 was chosen because there were too few infants in the -2 Z-score group for analysis. A significant regression equation was found (F=2.4, P=.0001, n=37), with an R^2 =.73. Total cases of fever (F=17.4, P=.0001) was found to be inversely correlated with Zwfl scores, and total occurrences of diarrhea (F=3.3, P=.02) was still found to be positively correlated with Zwfl. It is unclear

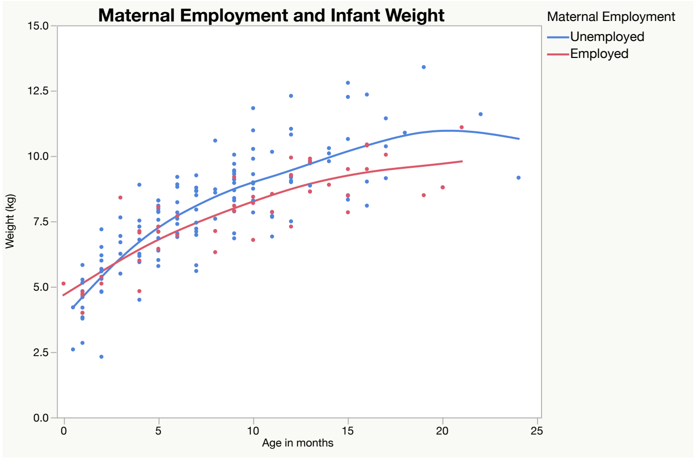


Figure 55: Maternal employment and infant growth curves.

AGP of infants of employed and unemployed mothers from birth until 2 years of age.

why increased numbers of diarrhea would contribute to higher Z-scores in both UGP and the general population of infants.

Q2H2: To explore the broader social, behavioral or ecological factors that influence growth outcomes (measured by Zwfl), a standard multiple regression was performed related to these broader factors. Preliminary analyses were performed to ensure there is no violation of the assumption of normality, linearity and multicollinearity. A significant regression equation was found ($F = 7.5$, $p = .0001$), with an R^2 of .37. Some of the significant health variables were premature birth ($F=4.0$, $p=.02$), and medical complications at birth (indicating a longer initial hospital stay, more medical interventions and lower rates of breastfeeding) ($F=6.9$, $P=.009$). Unsurprisingly, other socioeconomic and environmental factors including maternal employment ($F=5.15$, $P=.02$) (see Figure 55 and Figure 56), socio-economic status (SES) ($F=2.7$, $P=.04$) and the Household Microbial Ecology Index (HMEI) ($F=17.75$, $P=.0001$) (see chapter three for more details on the index) were found to be a significant predictor in this model of Zwfl. Interestingly, gastrointestinal illness, birth mode, gender, and early feeding practices were not found to be predictors of Zwfl.

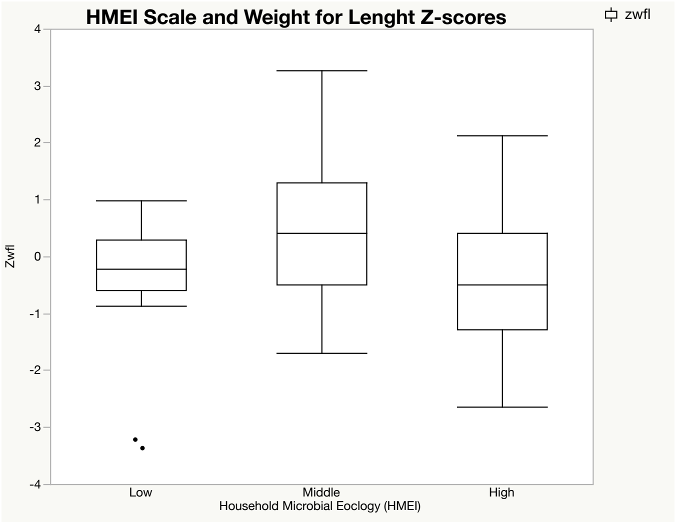


Figure 57: HMEI scale and Zwfl.

Children from either low or high HMEI households have significantly lower Zwfl scores (F=9.09, P=.0002).

Upon closer examination, children from households that have middle ranges of HMEI are significantly higher Zwfl for their age (F=9.09, P=.0002), compared to both the low HMEI group (P=.019), and the high HMEI group (P=.0003) (see Figure 57). Moreover, this trend holds across the four age groups, indicating that there is likely a healthy medium in household sanitation and cleanliness associated with growth outcomes.

Q2H3*: Formula fed and mixed-fed infants will weigh more and have higher rates of illness than breastfed infants.*

Infants who were formula fed gained more weight than those who were mixed or exclusively breast-fed (F=5.8, P=.0037), with mean weights at 9.16 kg, 7.69kg and, 7.99kg respectively (at 0-3 months). Moreover, after controlling for age, infants who were currently breastfeeding weighed less than those that were no longer breastfed (F=11.26, P=.001), although Zwfl scores were not significantly different among the feeding groups. Finally, breastfed infants were found to have lower rates of upper respiratory and GI illnesses, fevers, and overall sums of illness and antibiotic rates (see Table 34). Thus, contrary to the literature, formula-fed infants in this community weighed more, despite experiencing higher rates of illness.

The finding that formula-fed infants weighed more in the first months of life is not surprising given the literature (Dewey, 1998; Dewey, 2001) that shows weight-for-age z-scores (Zwei) in breast-fed infants tend to follow a downward trajectory beginning around 2–3 months in comparison to the CDC/NCHS growth reference of mainly formula-fed infants (Kramer et al., 2004), indicating a relative deceleration in

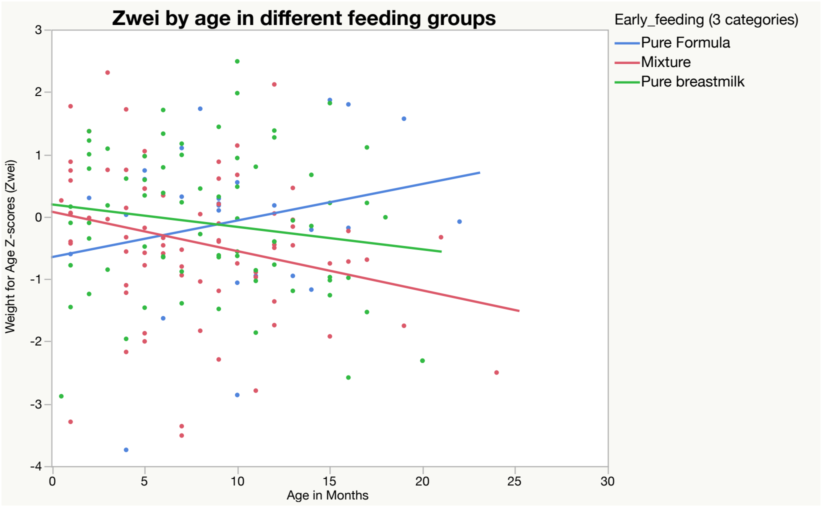


Figure 58: Feeding practices influence Zwei scores.

Breastfed and mixed fed infants show a deceleration in weight gain over time, whereas formula fed infants show an acceleration.

Average Rates of Illness Among Early Feeding Groups

Early Feeding	Upper Respiratory	GI	Fever	Sum Illness	Total ANTBX
Breast Fed	1.58	1.08	.54	3.34	2.26
Mixed Fed	2	1.33	.61	4.13	2.50
Formula Fed	2.32	1.39	.64	4.46	2.89

Table 34: Average rates of illness among early feeding groups.

Breastfed infants have lower rates of all type of illness. They also consumed fewer antibiotics on average than formula fed and mixed fed infants.

weight velocity. Interestingly, mixed fed infants in the current study showed an even steeper decline than the breastfed infants (Figure 58).

The current study overall revealed a more dramatic difference between breastfed and formula-fed infants than has been reported in infants of affluent countries. Formula-fed children of the current study were 670 g heavier than breastfed infants at 9 months and 1710 after 12 months, compared to Dewey's (1998) study which found formula-fed infants who were 400 g heavier than infants breast-fed at 9 months and 600–650 g heavier than infants breast-fed at 12 months. This early weight gain may place certain infants on obesogenic trajectories and possibly can help explain the explosive rates of obesity and chronic disorders currently observed in El Salvador and other Central American Countries (Aschner et al. 2011; Filozof et al. 2001).

Taken together, these analyses indicated evidence in favor of **Q2H3**: formula-fed infants tend to be heavier than their breastfed and mixed-fed counterparts, and accelerate in growth trajectories rather than decelerate as the mixed and breastfed infants did. Moreover, the mixed-fed infants rather than the breastfed were the lowest weight group, while formula-fed infants had the highest rates of infection and were treated with the greatest doses of antibiotics of the three early feeding groups. These findings are visualized in model Figure 59.

These analyses led me to question, what microbes were inhabiting the formula-fed infant's gut and how were they were different than the breast-fed infant's gut? The sample size was insufficient to run analyses on 0-3 months exclusively formula-fed infants, thus at At 4-7 mo, a significant regression equation was found (P= .013), with an R^2 of .19 when investigating Zwfl. This equation found *Blautia, Enterococcus,* and Enterobacteriaceae were positively correlated with Zwfl, while *Clostridiales* was negatively associated with growth. At 12+ months the significant equation was (P=.019), R^2 of .22, and only *Bacteroides* and *Enterobacteriaceae* were positively correlated with Zwfl, while Clostridiales, *Enterococcus* and *Blautia* were negatively associated with growth. Over the first

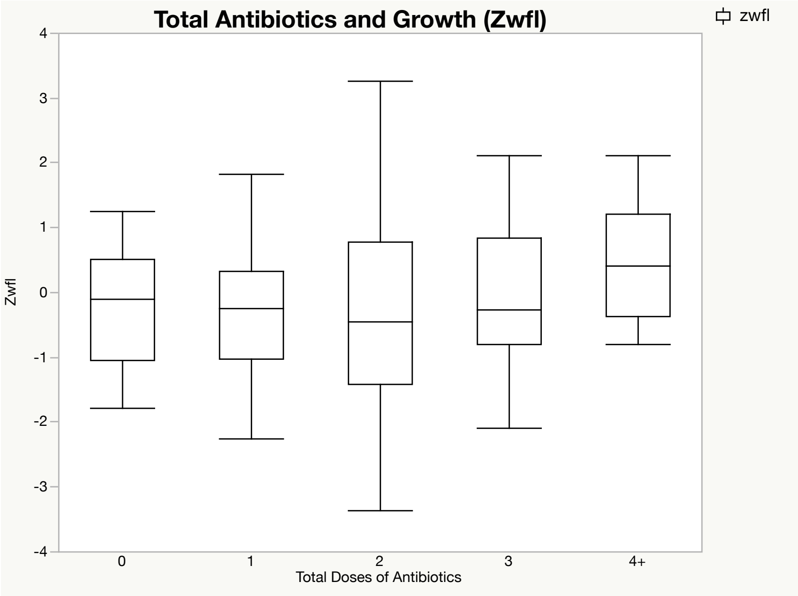


Table 35: Antibiotics and growth outcomes (Zwfl)

Infants who were administered 0-2 doses of antibiotics weighed significantly more than infants who were given 4+ doses.

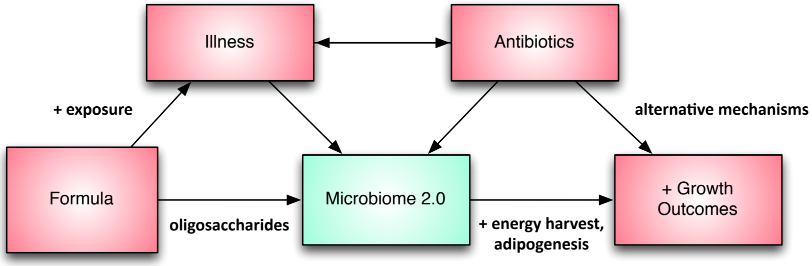


Figure 59: Infant feeding patterns, health and growth outcomes.

Pathways by which formula feeding alters the microbiome, thereby increasing weight gain and growth in early infancy.

year, Enterobacteriaceae was consistently found to be both prevalent in this population, and significantly associated with growth in formula-fed infants. *Clostridiales* was consistently found to be negatively correlated with growth in FF infants.

Q2H4*: Increased antibiotic usage should lead to greater infant growth/weight gain.*

Upon examination of the relationship between antibiotics and weight gain, after controlling for age, I found that infants who had not been given any antibiotics gained less weight than those who had been given 4 or more doses (F=4.5, P=.0019) (see Table 35). Moreover, children over one year of age who had taken 4 or more doses of antibiotics weighed significantly more than those who had taken 0-2 doses (F=3.3, P=.02). These results held when examining Zwfl: Infants who had been given 2 doses of antibiotics by the end of the third phase of the study were significantly smaller than those who had taken 4 doses of antibiotics (F=2.9, P=.02). These results are in alignment with current literature on the issue (Trasande et al. 2013; Saari et al. 2015; Schwartz et al. 2016).

Discussion

The human infant is under strong selective pressures to meet urgent energetic and nutritional needs of their growing bodies and brains during these first months of postnatal development. Pathogenic assaults in early months of life can further exacerbate these early energetic challenges by diverting resources from growth to immune maintenance, often causing disturbance in growth and development. Human growth is thus a key biomarker that not only indicates past and present health outcomes, but also future health potentialities. The pace at which infants grow, the types of fat or muscle that they acquire and at what time, can help determine their acute and future health.

Meanwhile, emerging evidence linking the development of the intestinal microbiome to early growth outcomes (Thompson 2012; Subramanian and Smith 2006; Blanton et al. 2016), supports the possibility of utilizing growth also as a biomarker to evaluate the "health" of a developing gut microbiome (and vice versa). Studies in human infants and mice suggest that successional microbiota development is optimized to satisfy the different growth needs of the infant at different stages of development**.** These critical understandings highlight the importance of examining the interconnected relationships between host growth patterns and microbial development, as well as the ecological and social variables that obstruct or support these evolutionary relationships.

This chapter explores the premise that microbiota influence host metabolism and immunity, thereby influencing growth phenotypes, but also that growth phenotypes can help describe the parameters of a "healthy," population-specific infant microbiome. For example, analyses revealed that alpha diversity associated with growth outcomes may be an important biomarker for a healthy infant gut, albeit age dependent. There was a growth-dependent range of alpha diversity in the first few months of life, where too low may inhibit growth outcomes, and too high may support overweight outcomes. This trend shifted at

12+ months, where AGP infants had the highest α-diversity compared to the other two groups. These results lend evidence to the hypothesis that contrary to the adult microbiome, α-diversity in the first few months optimally should fall within a narrow range (Sheets and Worthman 2014). This hypothesis suggests that a more vertical (from mother to infant) exposure would be favorable during the infants' immunologically vulnerable period. Yet by 12+ months the microbiome begins to resemble an adult profile and diversity can lend to a healthier, more resilient microbiome (and an ecologically sourced and regulated immune system) (McDade 2005).

Assessing weight gain and growth measures in infants of average "healthy" growth phenotypes, I found an expected assortment of microbes. In the early months, *Bifidobacterium*, *Bacteriodes*, *Faecalibacterium*, *Lactobacillus,* and *Veillonella* were positively correlated with Zwei. These associations resemble recent studies that found the association of these milk-degrading microbes to be strongly associated with healthy gut development and infant growth (Blanton et al. 2016; S. Subramanian et al. 2014). Also, as infants reached one year of age, *Bifidobacterium*, *Lactobacillus* and other microbes singularly associated with milk digestion became less important, and other taxa including *Prevotella*, *Akkermansia* and *Megasphaera* became more significantly related to measures of growth (Zwei and Zwfl). Although the exact mechanisms by which these gut microbes communicate metabolically with other tissues remain to be defined, what is clear is that microbes associated with growth successionally change over the first year.

There were other surprising outcomes when infants were segregated into growth-phenotype groups for analytical purposes. In contrast to the literature, a late blooming, yet keystone genus of the human gut, *Faecalibacterium* was found to be an important microbe associated with AGP infants. This was true even in the first months of life when the bacterium was at relatively low counts, and remained significantly associated with growth into the second year of life. Moreover, the OGP group had the highest levels of these bacteria (see Figure 50), lending further evidence that this bacterium is a strong promotor of growth in this population.

Lactobacillus was another key growth-related bacterium, although it was found in surprisingly short supply in this population. Lactobacillus was found to be growth-promoting bacteria in UGP infants only by months 12+, indicating a delayed colonization pattern in these infants. This finding agrees with current studies that suggest undernourished infants tend to have an immature gut microbiome (Subramanian et al. 2014). Also interesting is the UGP group had the lowest levels overall of this bacterium at all age points. Variables that seemed to encourage the colonization of this bacterium were breast-feeding and the number of different types of animals within a household. The importance of these bacteria to growth within this population and the numerous studies describing its probiotic effects highlight the surprise of finding such low levels in the study population and will require future research to better understand the reasons for such low levels. Given that Subramanian et al. (2014) ranked this bacterium 3 of 25 species as a taxonomic biomarker for healthy gut development, and the literature is replete with reports on the anti-inflammatory and probiotic effects of this bacterium, future research is required to better understand the functional effects of this low prevalence. Intra-population and cross-cultural research should reveal population differences, while developmental ecology research can explore proximal reasons for the drastically low levels in this and possibly other similar populations. Finally, it is possible that these bacteria simply are excluded based on functional redundancy, but further exploration is required.

The second research question shifted the analytical lens from growth associated microbiota to explore the macro and social factors that influence growth outcomes. From a socio-ecological perspective, two factors and their effects on growth require mentioning. The first was that infants of mothers who worked in either the formal or informal marketplace were significantly smaller than infants with mothers who were unemployed. Paradoxically, formula-fed infants were significantly heavier than their breastfed counterparts, and seemingly infants with working mothers would be more likely to be formula fed. However, many of the infants with working mothers were mixed-fed infants, and this feeding category was found to have the lowest Zwfl scores of the three feeding groups. Upon further investigation, I found that while infants of working mothers had significantly lower Zwfl scores in all three SES levels, the difference was most pronounced in the low SES group (F=12.7, P=.007). This finding is less surprising given the fact that the price of infant formula for a month is equivalent to a middle-income mother's monthly salary. Lower SES households rarely have refrigeration, making breast-pumping problematic. These circumstances lead to the poorest of caretakers feeding infants watered-down formulas or alternatives such as powdered cow's milk and in one case orange juice. A deeper understanding however, of why this effect holds across the SES levels requires investigation into feeding and caretaking practices that is beyond the scope of this chapter, although it is an intriguing finding that may have broad-sweeping and complex socio-political ramifications.

The second factor that deserves attention, was the finding that infants who are raised in highly sanitary or highly unsanitary households have lower Zwfl scores than those in the middle range group. This outcome held across the age groups, suggesting a strong trend. It also highlights the need for further research into how proximal social, ecological and developmental influences on growth outcomes via early microbial development.

Finally, in agreement with the literature, formula-fed infants were found to be heavier than their breast- and mixed-fed cohort. In fact, formula-fed infants experienced an acceleration of growth, compared to the deceleration that mixed and breast-fed infants experienced. Formula-fed infants also suffered the highest rates of illness. Given the extensive literature documenting the heavy costs of illness on infant growth patterns, this is a highly paradoxical relationship. One clue, however, may be the finding that infants who are administered 4+ antibiotics showed higher Zwfl scores than those given only 2 doses. FF infants were administered more antibiotics on average than BF or MF infants. Thus, it is possible that FF infants became ill more often, were prescribed more antibiotics, and when they reached a "threshold," their microbiome may have been altered metabolically, effectively increasing growth despite high rates of illness. This population was under the care of a diligent public health promotor who visited with every infant on a monthly basis. This likely resulted in more acute illness rather than long-term subclinical conditions that would have more of an effect on infant growth outcomes.

Another possible and complementary explanation may be the direct influence of formula on the microbiome. FF infants do not receive important maternal immune factors that help to prevent illness, but also breast-milk provides a steady flow of commensal organisms that take up residence in the infant gut, competitively excluding pathogenic microbes. Critically, breastmilk contains sugars called oligosaccharides that feed specific types of vertically inherited bacteria. While complex oligosaccharides are abundant in human breastmilk (between 5 and 8 g/liter), bovine-based formulas contain extremely low levels, with 3'-sialyllactose (3SL) being a main component (Kunz et al. 2000). High levels of 3SL in formula helps select for bacteria from the Enterobacteriaceae family, which includes species that exhibit a wide range of properties, varying from probiotic (Kruis et al. 2004), to pathogenic and deadly (Ron 2006). This family contains many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella* and *Shingella* among others that have been known to proliferate during disease and exacerbate intestinal inflammation. FF infants have been shown to harbor higher levels of *E. coli* and other pathogenic bacteria from the Enterobacteriaceae family (Penders et al. 2005; Palmer 2007).

In the current study, breastfeeding was associated with colonization patterns: FF infants harbored nearly double the relative abundance of Enterobacteriaceae that BF infants harbored (45.6% and 24.8% respectively) (P=.0004). MF infants also harbored significantly more of these bacteria than BF infants (P=.04), suggesting that even a partial diet of cow's milk formula is enough to encourage early blooms of Enterobacteriaceae. These findings align with current research that shows the protective nature of human breastmilk. Maternal breastmilk sIgA recognize Enterobacteriaceae and coat the bacteria (Tsuruta et al. 2010), hampering their proliferation in the gut (Palm et al. 2014), in part helping to explain increased pathogenesis in MF and FF infants.

The exact mechanisms by which Enterobacteriaceae affect growth are unclear. It may be that these microbes participate directly in degrading and adipogenesis, or it may be that their pathogenic activities encourage antibiotic administration which ultimately alters the microbiome to an obesogenic profile. Either way, I found that beginning at 8 months, and continuing into the second year, Enterobacteriaceae was positively correlated with Zwfl scores in the general population as well across the growth phenotype groups. Beginning earlier than that 0-3 months, it was positively correlated with Zwei scores, suggesting its key import in these populations. Furthermore, in exclusively FF infants at 4-7 months and again at 12+ months, Enterobacteriaceae was positively correlated with growth. Over the first year, Enterobacteriaceae was consistently found to be both prevalent in this population, and significantly associated with growth in formula-fed infants.

A primary limitation of this study was that small community population size meant that infant ages at baseline ranged from 0-8 mo. These age differences complicated longitudinal analyses, whereas a typical cohort study would have more precise longitudinal analyses of changes based on specific ages. Also, limited sample size made certain statistical analyses impossible when dividing infants into subgroups. Thus, in some cases it was difficult to tease apart different sources of variation and explore likely confounding variables. At the same time, this study followed nearly 100% of the infants under one year of age in this community, which provided a comprehensive exploration of the range of issues for this community of infants at all sampled ages from a cross-sectional and in some instances, longitudinal perspective.

Another limitation was the use of fecal samples to represent the gut microbiome, which may not accurately represent the actual colonization patterns of the large intestine (Zoetendal et al. 2006). As most other studies that focus on gut microbiome, the current study utilized infant fecal matter to represent the microbial composition of the large intestine. Unfortunately, as with any DNA-based, culture-independent study that does not discriminate between live and dead bacteria, the number and identity of bacteria detected in this studies may not represent actual bacterial counts, and should be interpreted with some caution (Zoetendal et al. 2006).

Limitations aside, this chapter explored the successional and metabolically active nature of the infant microbiome. What microbes inhabit the gut at specific times during development help shape growth outcomes. Growth outcomes in turn, can help us turn the lens onto the health of a gut microbiome. Thus in all, this chapter contributed to understandings of the successional nature of human-associated, microbial development. It identified metabolically active microbiota, as well as broader variables that contribute to different growth phenotypes. While this population did not show a sub-clinical disease burden that inhibited growth outcomes, high rates of illness instigated a high level of antibiotic administration in infants. Given recent literature on antibiotic administration and obesogenic outcomes (Cho et al. 2012; Cox et al. 2014; Blaser 2014; Dominguez-Bello and Blaser 2015), it is likely that high levels of consumption during this early period of development will show up in metabolic and immune disorders in later childhood and/or adulthood. Finally, this chapter explored multiple ways of examining and determining what a “healthy” infant microbiome may look like in this given population. Thus, by exploring growth in relation to microbial colonization patterns we can begin to define what a “healthy” versus a dysbiotic microbiome may look like in distinct populations, and through these insights we can develop locally-appropriate public health policies and interventions to alleviate and prevent human suffering.

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CHAPTER 5: FUNCTIONAL INDEX OF RELEVANT TAXA

Introduction

The early colonization of the infant gut microbiome is a rapidly growing field of study. There are many biological, genetic, cultural and ecological factors involved in the early development of the microbiome, rendering it a complex field of study. One way to address this complexity is to explore the microbial diversity within one ecological setting. This chapter capitalizes on the ecological variation within this Salvadoran population to understand the dynamics of early infant colonization and maturation of the gut microbiome in the first year of life.

The purpose of this chapter is to act as a reference guide of relevant bacterial taxa. For each genus, class or family taxon, a general description is provided, followed by a brief review of current and relevant literature. Next, a description of findings related to the development of each taxa are reported. Data on outlier infants who have microbial ranges higher (and in some cases, lower) than the predictive range will be explored. These outlier analyses help describe the functional benefits or hindrance of each bacterial genera at specific stages of development, as well as to describe a more holistic exploration of infant microbial ecology. Finally, the discussion explores the patterns among these microbes as they successionally colonize the infant intestinal tract. The final section categorizes the bacteria in multiple ways to better assess the functional and developmental timings of various microbes.

Akkermansia

Akkermansia is a Gram-negative, oval-shaped, non-motile strict anaerobe. The genus contains one known species*, A. muciniphila,* and is a common bacteria that takes residence in, draws nutrition from, and contributes to the regeneration of the mucosal layer in humans (Egshatyan et al. 2016). The bacterium is thought to initiate a positive feedback loop between host and microbe (Belzer and de Vos 2012): once in residence, mucosal degradation stimulates mucus production, which in turn supports bacterial growth. Because of its abundance in healthy mucosa and the inverse correlation with several disorders including obesity (Zhang et al., 2009; Santacruz et al., 2010), IBD (Png et al., 2010), appendicitis (Swidsinski et al. 2011), and children with autism (Wang et al., 2011), members of the genus *Akkermansia* have been suggested to be biomarkers for a healthy intestine (Png et al. 2010; Swidsinski et al. 2011). The bacterium is naturally present in the human digestive tract at 3-5%, but has been seen to fall with obesity and other inflammatory conditions.

A recent experimental study suggested that *A. muciniphila* reduces fat mass gain and increases glucose metabolism when administered to mice with diet-induced obesity (Everard et al. 2013). Another recent study showed an association between *A. muciniphila* abundance, insulin sensitivity and healthier metabolic status in overweight/obese adults (Schneeberger et al. 2015). Research in humans shows that healthier subjects are those with high *A. muciniphila* abundance and gut microbial richness (Dao et al. 2016). Finally, it has been suggested that through thickening the gut wall, the bacterium helps to block the absorption of food from the body (Passel et al. 2011).

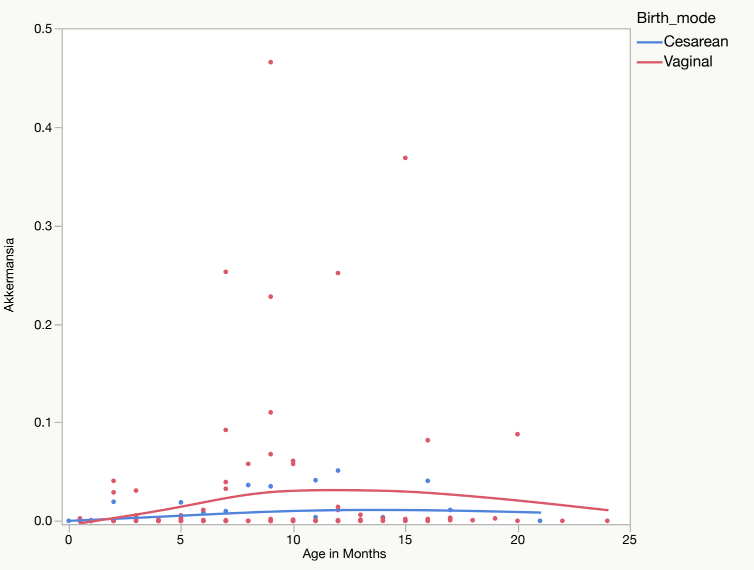

Figure 60: Patterns of *Akkermansia* colonization over time and by birth mode.

Vaginally birthed (VB) infants show increased relative abundance of Akkermansia.

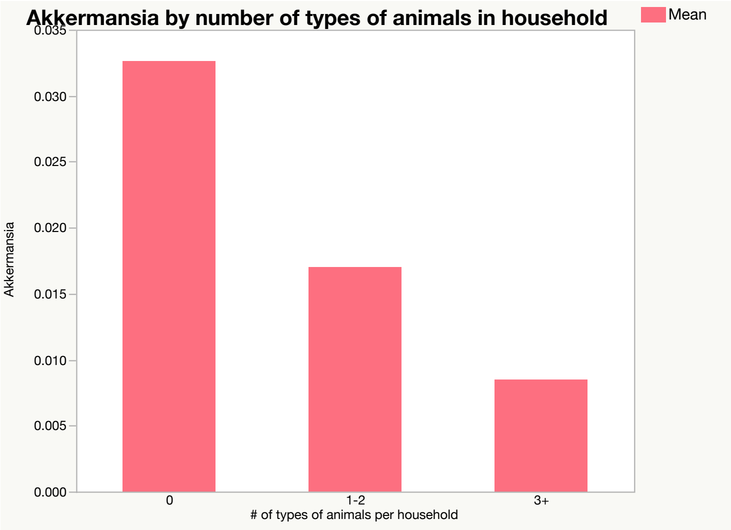

Figure 61: Fewer animals in household predicts higher levels of *Akkermansia*.

Infants that live in households with more types of animals have on average lower relative abundance of Akkermansia in their gut microbiota.

The presence of *A. muciniphila*-like bacteria was found in 1 month old infant fecal samples, and the number increased rapidly by the first year (Figure 62). The numbers of bacteria related to *A. muciniphila* almost doubled in the age period ranging from 6 to 12 months (M. Carmen Collado et al. 2007). These data are in agreement with earlier observations (Midtvedt et al. 1994), which reported the establishment of mucin-degrading microbiota in children from birth to the age of 2 years. The establishment of mucin-degrading bacteria was reported during the first months of life and is completed when the children are around 2 years old (IBID). These data indicate that *A. muciniphila* is colonizing the intestinal tract in early life and develops within a year to a level close to that observed in adults. At the same time, there is little research that documents the colonization patterns of these bacteria.

Study findings

Similar to Collardo et al (2007), study infants showed low levels of *Akkermansia* in the first months, however an increase only after 6 months (Figure 62). Interestingly, birth mode seemed to affect the early colonization of *Akkermansia* in study infants (Figure 60). Cesarean delivery (CD) infants showed a delayed colonization pattern of *Akkermansia* and did not catch up to infants born by vaginal delivery (VD) until the end of the first year of life (8-11 months), a trend reflected also in the feeding groups. Most striking, CDFF infants never acquire levels above .33% throughout their first two years of life. This highlights the compounded importance of both birthing practices and

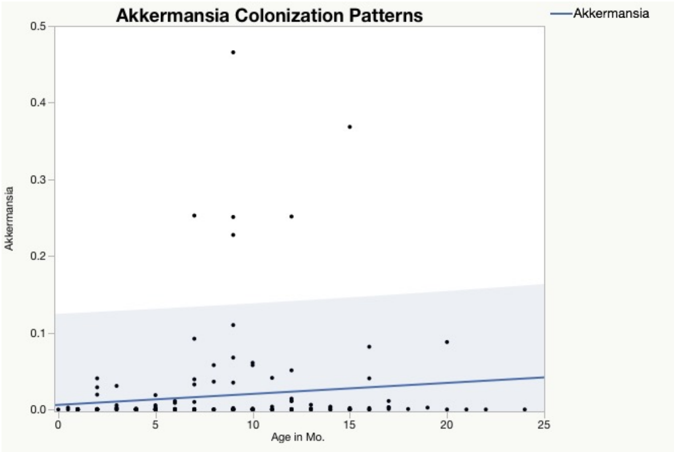

Figure 62: Akkermansia colonization patterns.

Akkermansia increases in abundance over time. increasing by month 7.

early feeding during the first years of microbiome development (sample size was too small to test).

Moreover, environmental and socio-ecological factors may influence the development of these bacteria. Households with more types of animals had lower average relative abundance of bacteria than did households with fewer types of animals (see Figure 61). Additionally, there was a trend towards higher levels of *Akkermansia* when infants had lower levels of α-diversity, although the difference was not statistically significant. This lends evidence to an age-dependent range of alpha diversity (ADR-a) that may encourage specific, vertically transmitted microbiota in the early months. Thus, households with the most diversity of animals in the household may not support the microbial colonization of this bacterium. Finally, levels of *Akkermansia* were positively correlated with growth at 12+ months.

High levels of this bacterium did not seem to affect infants adversely. Only five infants had levels above the predictive range, and subject number 7 had high levels at 12 mo. and at 15 mo. but levels had dropped to 0% by 19 mo. The other four infants with high levels of Akkermansia were between 7-9 months of age, and all were relatively healthy during the interviews and collection phases. This group had a range of Zwfl scores, although subject 7 was above the 95% percentile for growth (Pwfl), which is surprising given the literature on Akkermansia being protective against obesity/overweight.

Key words: Anti-inflammatory, healthy biomarker, protective against obesity and inflammatory conditions.

Bacteroides

Bacteroides is a genus of Gram-negative, obligate anaerobic bacteria. *Bacteroides* species are non-endospore-forming bacilli and may either be motile or non-motile, depending on the species (Madigan and Martinko 2006). These bacteria are variably abundant residents of the neonate gut, but by the first year of life they are consistently present in the gut (Palmer et al. 2007). Like *Bifidobacterium*, certain species of *Bacteroides* including *B. thetaiotaomicron, B. fragilis* and *B. vulgatus* are able to metabolize human milk oligosaccharides (HMO) with high efficiency (Marcobal et al. 2010). Dissimilarly, however, these *Bacteroides* species are also well adapted to use a multitude of both dietary polysaccharides and host-derived glycans (e.g., mucus) due to specialized machinery encoded by multiple linked genes called the polysaccharide utilization loci (PULs) (Martens et al. 2008). *Bacteroides* species have a wide variety of PULs and each appears to be specialized in the use of a particular class of carbohydrates (Martens et al. 2008; Sonnenburg et al. 2010). The ability of *Bacteroides* to utilize HMO suggests that specific PULs within these *Bacteroides* genomes are involved in HMO use. Moreover, *Bifidobacteria* have been shown to stimulate the diversity of sugars that *Bacteroides* can degrade for nutrients and energy (Martens et al. 2008).

Bacteroides are also involved in immune regulation: *Bacteroides* have been shown to induce several host genes that are involved in innate immune response of the infant. Specifically *Bacteroides fragilis* is able to enhance the function of various T cells (Turroni et al. 2008).

Bacteroides are bacteria that are affected by socio-cultural factors in developing countries, including birth mode and antibiotic administration. The colonization of *Bacteroides* is often delayed (up to a year) in Cesarean-delivered European infants (Grönlund et al. 1999). By contrast, in Nigeria, both vaginally and Cesarean–delivered infants harbor *Bacteroides* within a week in most cases (Rotimi et al. 1985). It has also been shown that *Bacteroides* spp. are not usually re-established after antibiotic treatment (Bennet and Nord 1987). These findings suggest that *Bacteroides* are a genera that are particularly sensitive to biomedical practices at birth and beyond, evidenced by earlier studies in Swedish infants showed significantly higher levels of bacteria (Bennet and Nord 1987; Lundequist et al. 1985) than more recent studies (Laursen et al. 2016; Ingegerd Adlerberth et al. 2006).

Moreover, Vael et al. (2011) found that BMI z-scores between 1 and 3 years old were positively associated with levels of *Bacteroides* at 3 and 52 weeks of age and negatively with *Staphylococcus* at 3 and 26 weeks of age, controlling for infant feeding and several other risk factors for higher BMI.

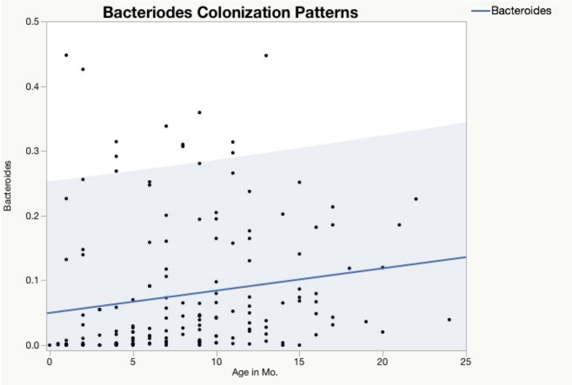

Figure 63: *Bacteroides* colonization patterns.

This bacterium is an early colonizer in this population and increase in the general population over time.

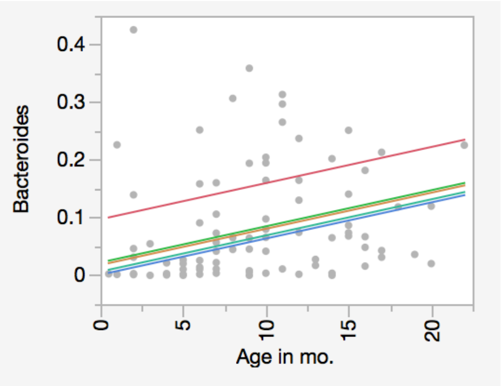

Figure 64: *Bacteroides* by age and antibiotics.

Infants who have not been treated with antibiotics have significantly higher levels of *Bacteroides* than infants who have received 1-4+. Red represents 0 antibiotics.

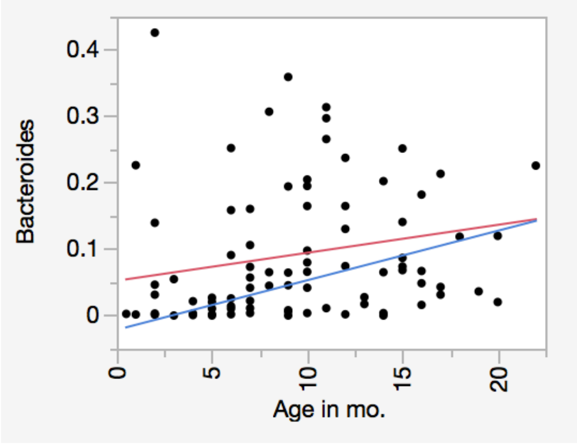

Figure 65: *Bacteroides* colonization by age and early feeding.

Red represents BF, and blue represents FF infants. Although not statistically significant, early feeding appears to affect levels of *Bacteroides* in the infant gut in the first months of life.

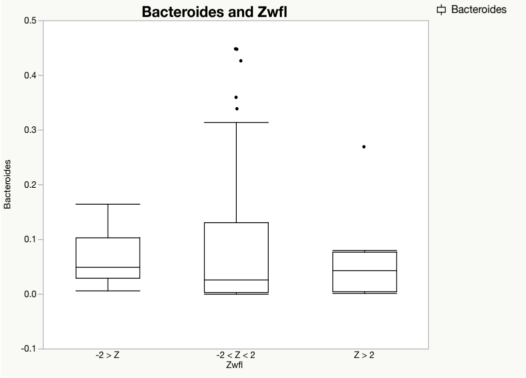

Figure 66: Bacteroides and Zwfl.

Although average levels are not significantly different among growth phenotype groups, the AGP (middle group) have the largest range.

Study findings

The current study found no significant difference among the birth modes and early feeding. However, similar to Vael et al. (2011), *Bacteroides* were found to be positively correlated with all four measures of growth in the first year of life, particularly after 12+ months. Curiously, in the overweight growth phenotype (OGP) individuals, *Bacteroides* was negatively correlated with growth factors (see Figure 66).

The current study also found that antibiotic treatment significantly affected colonization patterns of *Bacteroides* in infants. Those that did not receive antibiotics had significantly higher levels of *Bacteroides* among all age groups (P=.0033) (see Figure 64), aligning with other research that showed that *Bacteroides* spp. were not usually re-established after antibiotic treatment (Bennet and Nord 1987). These findings suggest that *Bacteroides* are a genera that are particularly sensitive to biomedical practices at birth and beyond. Among the infants who had higher than the predictive range of these microbes, only 2/12 were CD, and all infants were either BF or MF, showing that these early factors may be critical to hosting high levels of this bacterium. None of these infants seemed to have adverse reactions to high levels of these bacteria.

Key words: metabolizes HMOS, PULs, sensitive to antibiotics.

Bifidobacteria

Bifidobacteria are a genus of gram-positive, non-motile, often branched anaerobic bacteria. They are among the most common bacterial members of the infant intestinal microbiome. The most common *Bifidobacterium* species in infants are *B. infantis*, *B. breve*, and *B.* longum. *Bifidobacteria* are transferred from the mother during delivery (Mueller et al. 2015; Jost et al. 2014) and therefore are more frequent in vaginally compared with Cesarean–delivered infants in the first weeks of life. In a matter of weeks, however, cesarean–delivered infants typically acquire *bifidobacteria,* although the sources have yet to be discovered. *Bifidobacteria* are relatively tolerant to ambient oxygen (Simpson et al. 2005), which may facilitate their spread by socio-ecological means. Moreover, in much of the literature, early feeding does not seem to affect levels of the bacterium, as species colonize in great numbers in the infant digestive tract, regardless of whether the infant is breast-fed or formula-fed (Adlerberth et al. 1991). Infants born in wealthy western populations seem to be less affected by these early care practices.

Bifidobacteria is a common genus within the infant gut microbiome also because of their ability to break down breast-milk Oligosaccharides such as N-acetylglucosamine, glucose, galactose, and certain glycoproteins contained in the milk. This affords these bacteria the ability to obtain energy and nutrients for host and bacterial growth. *B. infantis* for example, is a bacterium unique to the infant's digestive tract (Matsuki et al. 2003), and prefers glucose over other oligosaccharides due the sugar being easily metabolized and highly abundant (Marcobal et al. 2010). *B. infantis* attach to the epithelium or entrap themselves in the mucousal layer of the epithelium, where they can focus on their metabolic activities. These bacteria breaks down and utilizes essential components of ingested food with the assistance of intestinal peptidases such as alpha-glutamyl transpeptidase, a minopeptidase, oligoaminopeptidase, and carboxypeptidase (Berseth et al. 2006). Effective digestion by the intestinal peptides and hormones within the environment, allows for the microbe to decrease energy expenditure, while increasing energy harvest.

Another common species *B. longum*, was found in Malawian and Bangladeshi infants to positively affect growth. This species was an early colonizer with its highest mean relative abundance at 5 months of age (Blanton et al. 2016). The abundance of complex oligosaccharides within milk attracts both mutualistic mucus-adapted species and HMO-adapted *bifidobacteria* to the infant intestine that likely facilitate both milk and future solid food digestion (Marcobal et al. 2011), supporting both growth and also likely immune properties of the infant gut.

Increased colonization of *Bifidobacterium* in the large intestine, and its interaction with *Lactobacilli*, results in enhanced carbohydrate fermentation (Wall et al. 2008). Fermentation results in an increased production of acetic acid, butyric acid, and lactic acid, which creates an acidic barrier against pathogenic bacteria. *B. infantis* interacts with *Lactobacillus salivarius* to exert immunomodulatory effects on intestinal immune cells that mediate host responses to flagellin and pathogens. They are able to modulate the intestinal epithelium by making Salmonella typhimurium less virulent as well as weakening flagellin-induced pro-inflammatory responses (Wold 1998). Both species interact to down-regulate the secretion of basal IL-8, but *B. infantis* specifically inhibits flagellin-induced IL-8 secretion. Flagellin serves as a key activator of pro-inflammatory responses to specifically Salmonella intestinal epithelial cell responses (Wold 1998). The major point to understand from this is that *B. infantis* interacts with *Lactobacillus salivarius* to modulate intestinal epithelial cell responses by limiting IL-8 secretion. While they are interacting to weaken pro-inflammatory responses, they may encounter other microbes such as *Bacteroides vulgatus* that activate pro-inflammatory gene expression in intestinal epithelial cells (Wold 1998).

Study findings

In alignment with the early studies, current study infants who were CD had significantly lower levels of *Bifidobacteria* than infants who were VD, before (P=.029, F=4.8) and after controlling for age (P=.03, F=5.2). Similarly, infants who were BF had significantly higher levels of *Bifidobacteria* than infants who were FF (P=.018, F=4.4). This was in direct contrast to Western populations who seem to be less dependent on breastfeeding for the colonization of this bacterium (Adlerberth et al. 1991). Antibiotics, were not significantly associated with colonization patterns, nor were some of the more socio-cultural factors including SES, maternal work, etc.

Bifidobacteria were significantly correlated with growth measures (Zwfl, Zwei) in the general populations, and most significantly among the 0-3-month age group. Upon further investigation, *Bifidobacterium* was significantly related to Zwfl in 0-3 month olds within the average growth phenotype (AGP), but not the under (UGP) or over weight growth phenotype (OGP) groups. As expected, *Bifidobacterium* and other microbes singularly associated with milk digestion became less related to healthy growth phenotypes over time, as the infant diet diversified. Thus, *Bifidobacterium* dominates during the early months, and functionally supports “healthy” growth. In this Salvadoran population, this bacterium is likely vertically selected through birth-mode and breast-feeding.

Five infants harbored levels of this bacterium that were above the predictive range of *Bifidobacterium*, and subject 17 had higher ranges at 3 months and again at 6 months. All but one of the infants were VD, and All were either BF or MF. Both subjects 17 and 18 simultaneously had high levels of *Bifidobacterium* and *Lactobacillus*, while all the other infants from this group had at least average levels of the *Lactobacillus*, highlighting the symbiosis of these two bacteria. Infants in this outlier group were generally healthy.

Assessing infants with low or undetectable levels of this bacterium revealed converse stories: they had some combination of cesarean deliveries, and mixed to formula feeding, antibiotics and multiple infections. Subject 53 for example, was breastfed for the first months of her life. Although she never had detectable levels of Lactobacillus, in her 2nd month, she had high levels of Bifidobacterium (26.3%) and Lachnospiraceae (24%). She was healthy per her mother. Towards the end of her 3rd month, the infant acquired an infection and had blood in her stool. She was given a series of antibiotics, and was sick on and off for the following two months. At 5 months, she had extremely high levels of Lachnospiraceae (32%), and then at 9 months she had extremely high levels of *Faecalibacterium* for her age (10.2%).

Key words: Metabolizes HMOs, marker of AGP “healthy” growth, early ADR-a microbe, supports immunity, anti-inflammatory, vertically selected in study population, synergistic with Lactobacillus.

Blautia

Blautia are Gram-positive, anaerobic bacteria. The functional significance of *Blautia* in the intestinal tract of infants is poorly understood. In studies of adults, *Blautia* has been associated with good cognition and decreased inflammation (Bajaj et al. 2012) likely due to the bacteria's butyrate-producing activates. *Blautia* levels are increased in healthy adults when compared to patients with liver disease and colorectal cancer (Chen et al. 2012). Studies in children and infants however are contradictory: in childr*en with type 1 Diabetes, as some studies find a decrease in Blautia in children with T1D (Murri et al. 2013), while Kostic et al. (2015) found that Blautia became more abundant in infants who* later acquired T1D. This later study considers *Blautia* to be a "pathobiont" (Chow and Mazmanian 2010), which are members of the commensal microbiota that have the capacity to behave as pathogens. A recent study indicates that the acquisition of specific strains of *Blautia* (in combination with other probiotic bacteria including *Roseburia* and *Coprococcus*) are associated with lactose tolerance in infants (Canani et al. 2016). The conflicting evidence on this genus illustrates the compounding complexity of the intestinal microbiota ecosystem, microbiome-host interactions, host life-history, and finally ecology-host-microbiome relationships. In other words *Blautia* may have probiotic or pathogenic effects on humans depending on microbial community interactions, during specific phases of the life-cycle, or in specific context-dependent populations.

Study findings

Although birth mode was not a significant factor for *Blautia* colonization in this population, infants who were exclusively breastfed had significantly lower levels of the bacteria than infants who were mixed fed (P=.01), and mixed fed infants had significantly lower levels of *Blautia* than those who were formula fed (p=.006), suggesting that breast-feeding may have a strong protective effect compared to formula feeding. Additionally, infants who were currently breastfeeding versus those who had been fully weaned had lower levels of the bacteria in their microbiota (P=.001). Subject number 24 was never breast-fed, and at nine months had an extremely high relative abundance of *Blautia* (38%), although his levels of the bacteria dropped to more normal ranges in subsequent months (0-10%). This subject was fed low-fat cow's milk rather than formula or breast-milk. This child suffered many gastrointestinal and upper-respiratory illnesses and allergic reactions throughout the study period. Other infants who were fed high levels of powdered cow's milk and who also had intestinal issues tended to have higher levels of *Blautia*, including subjects 7 and 9. Concurrent to high *Blautia* levels, these subjects also tended to have high levels of Clostridia, indicating a possible interaction among these pathobionts.

Moreover, upper SES levels had the highest levels of *Blautia* when compared to the middle SES group (P=.007), and the lower SES group (P=.002). Infants in households that did not contain animals had significantly higher levels of *Blautia* in their microbiota (P=.002). Infants of high HMEI households had significantly more of these bacteria than infants of middle HMEI households

(P=.017), and although not significant, infants of the low HMEI households had higher levels than those of the middle HMEI group, indicating a U-shaped pattern (see Figure 67). This suggests that households that were too clean and without animals in some way encourage the over-population of these bacteria.

Blautia is positively correlated with growth in infants in the general populations, specifically in infants 4-7months, and again at 12+ months. However, among average growth phenotype (AGP) infants and overweight growth phenotypes (OGP) infants *Blautia* was negatively correlated with growth at 0-3 months and 12+ months, respectively. Yet the bacterium was positively associated with growth in exclusively FF infants.

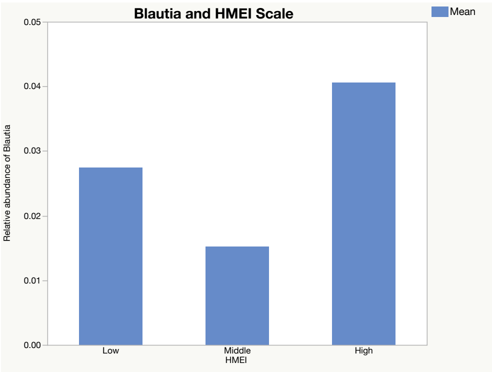

Figure 67: HMEI households and levels of Blautia.

Middle HMEI household have the lowest levels of Blautia, indicating that an average level of microbial exposure to this microbe may be advantageous (considering the hypothesis that moderate levels of this bacterium are preferred).

Taken together, these findings suggest that exclusive bovine-milk consumption (regular powdered milk and infant formula) is highly associated with high levels of *Blautia*, increased levels of illness, and may be in part responsible for obesogenic phenotypes of FF infants in this population. This bacterium, a known SCFA producer is likely selected for by a bovine-milk diet and in turn through pathogenic activities may encourage imbalance, increased antibiotic consumption and thus obesogenic profiles. Although exploring this population at the species and experimental level would help define understandings of these bacteria, evidence in this population indicates that high levels of this bacterium is a biomarker of an unhealthy gut, at least in the first months. Meanwhile *Blautia* may be a common member of the commensal community in BF infants.

Key words: pathobiont, controversial, context and age-dependent pathogenesis, bovine-milk selection

Clostridia

Clostridia are Gram-positive, spore-forming, obligate anaerobes. Many species of *Clostridium* are pathobionts, due to their ability to form toxins and spores (Nevas et al. 2005). Cesarean delivery and a formula-based diet has been shown to increase colonization of *Clostridia* in an infant's gut. *Clostridia* thrive in the anaerobic environment of the intestines, where they can multiply in great numbers and produce toxins (Nevas et al. 2005). However, if nutrient availability becomes low, or the environment becomes unfavorable, they can often survive through sporulation.

The most common *Clostridium* species found in an infant's gut is *C. difficile*. *C. difficile* can colonize in large numbers in the intestines, increasing the production of toxins. These toxins are a common cause of diarrhea in infants (Mutlu et al. 2007). A proliferation of *Clostridium difficile* can be life-threatening to infants, particularly those taking antibiotics. Antibiotics often target and reduce the colonies of potential *C. difficile* competitors, causing blooms of *C. difficile* and their production of toxins. Blanton et al. (2016) recently found that undernourished infants and mice were dominated by bacteria from the *Clostridium* genus.

Study findings

CD infants had significantly higher levels of these bacteria than did VD infants in the first 3 months (F=6.1, P=.018), although the effect disappeared after these first months. Early feeding alone was not a significant factor for these bacteria (even after controlling for age), although when these factors are examined together, effects may be amplified: CDBF infants had the highest level of these bacteria at 0-3 months (>11% compared to 2.8% in VDBF infants) (although CDFF infants could not be analyzed because the sample size of this group). Levels of this bacterium reappeared only after the first year of life in CDFF infants only, indicating lingering effects of birth-mode. *Clostridium* was not associated with illness, antibiotic consumption, socio-cultural variables such as HMEI and SES nor growth scores. These non-associations were surprising given that pathogenic activities associated with this bacterium, although the measures of illness in this study had substantial limitations (presented in the final discussion chapter).

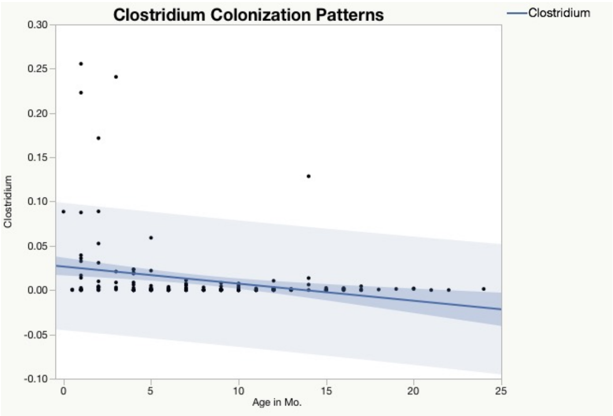

Figure 68: Clostridium colonization patterns.

Clostridium is an early colonizer that decreases by the 5th month of life.

Key words: pathobiont, birth-mode and early feeding sensitive, early colonizer

Clostridiales (order)

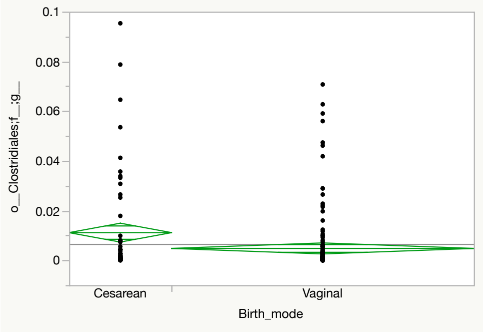

Figure 69: Clostridiales and birth mode.

CD infants had significantly higher relative abundance of Clostridiales when compared to VD infants.

Clostridiales is the order of Clostridium. Clostridiales species inhabit soil, the intestinal tract of humans, as well as the healthy lower reproductive tract of women. Azad et al. (2014) found that breastfed infants had a low to 0 prevalence of this order of bacteria until after the first year of life.

Study Findings

Consistent with previous studies, breastfeeding promoted a decreased abundance of Clostridiales, where FF and MF infants show an early increase in abundance beginning the 5th month of life (see Figure 70). From 0-7 mo. breastfed infants have significantly lower levels of this bacterium than MF infants (P=.0008), and than FF infants (P=.04). Likely this order is seeded at birth, as it lives in the vagina of healthy women. Thus delivery

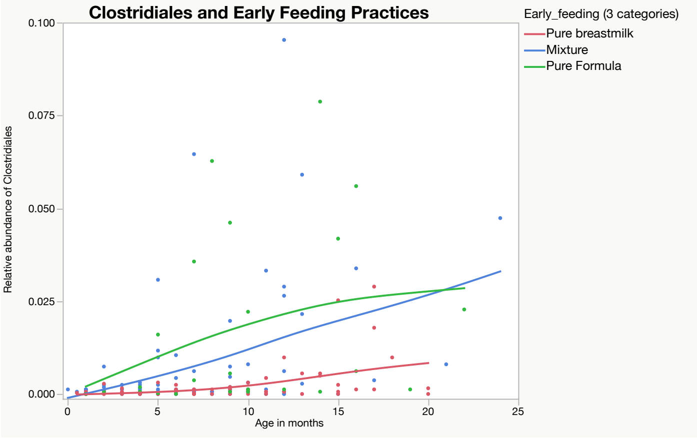

Figure 70: Patterns of Clostridiales colonization over time.

BF infants have a low to 0 prevalence of this order of bacteria until after the first year of life. FF and MF infants show an early increase in abundance after the 5th month of life.

significantly affected this colonization in infants: CD infants had significantly higher levels of bacteria than did VD infants (F=8.12, P=.005), even after controlling for age (P=.005) (see Figure 69). Subject 24 had relatively high levels of these bacteria at 9 months (4.9% compared to others who range from 0-1%). Subjects 7, 9, 24, and 70 also had high levels at the same time points that they had high *Blautia*. In UGP and AGP infants, Clostridiales were found to be positively correlated with growth, and negatively correlated with growth in the OGP group.

Key words: pathobiont, birth-mode and early feeding sensitive, breastmilk protective, >5 mo. colonizer

Corynebacterium

Corynebacteriaum is a genus of bacteria that are Gram-positive and aerobic. They are rod-shaped (bacilli), and in some phases of life they are club-shaped, which inspired the genus name (*coryneform* means "club-shaped"). They are widely distributed in the microbiota of animals, including the gut microbiota, and are mostly innocuous (Collins et al. 2004) although some species can cause

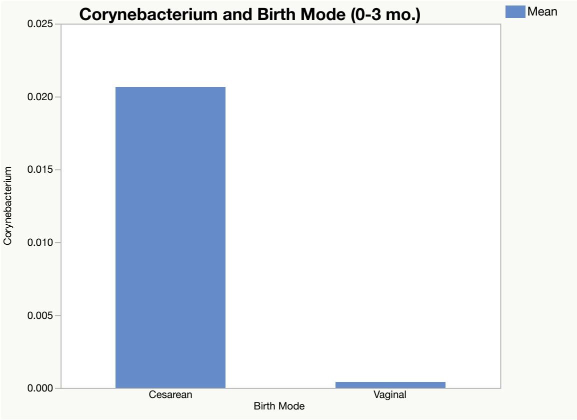

Figure 71: Corynebacterium in birth modes.

Although not significant, there seems to be more of this bacterium in C-section infants compared to vaginally born infants in the first three months of life.

human disease, including most notably diphtheria, which is caused by *C. diptheriae*. Thus, this bacterium can be classified as a pathobiont that occasionally can capitalize on atypical access to tissues (through open wounds), or weakened host defenses.

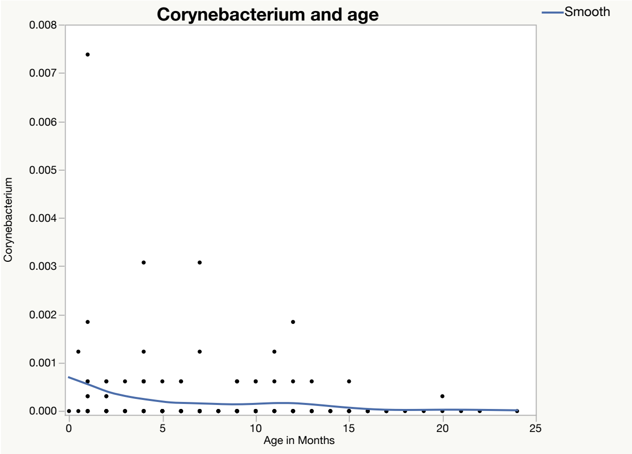

Figure 72: ***Corynebacterium*** **colonization patterns.**

These bacteria decrease in this population over time, reaching lowest levels by 16 months.

Four subspecies are recognized: *C. d. mitis*, *C. d. intermedius*, *C. d. gravis*, and *C. d. belfanti*. The four subspecies differ slightly in their colonial morphology and biochemical properties, such as the ability to metabolize certain nutrients, but all may be toxigenic (and therefore cause diphtheria) or not toxigenic. *C. diphtheriae* produces diphtheria toxin which alters protein function in the host by inactivating the elongation factor EF-2. This causes pharyngitis and 'pseudomembrane' in the throat. The diphtheria toxin gene is encoded by a bacteriophage found in toxigenic strains, integrated into the bacterial chromosome.

A low concentration of iron is required in the medium for toxin production. At high iron concentrations, iron molecules bind to an aporepressor on the beta bacteriophage, which carries the *Tox* gene. When bound to iron, the aporepressor shuts down toxin production (Nester et al. 2008) for toxogenicity is used to determine whether the organism is able to produce the diphtheria toxin.

Corynebacterium is likely more common in school-aged children of developing countries (Iizuka et al. 1980). These bacteria were also found to be primarily on the skin of mothers, passed primarily to infants born via C-section (Dominguez-Bello et al. 2010).

Study findings

This bacterium never reached levels above .08 %, and dropped off by the second year of life in study infants (see Figure 72). Although birth mode was not significantly related to the relative abundance of *Corynebacterium*, there certainly was a general trend in the first 3 months of life (P=.059) (see Figure 71). *Corynebacterium* was negatively correlated with growth (Zwfl) in from 0-7 months, and yet seemed to live as a non-pathogenic community member among average growth phenotype (AGP) infants at 12+ months. This bacterium was not associated with illness in infants, although it had consistently negative association with growth in the early months.

Key words: Pathobiont, inversely associated with growth markers in early infancy (0-7 months).

Enterobacteriaceae (family)

Enterobacteriaceae are a large family of Gram-negative bacteria, including species that exhibit a wide range of properties, varying from probiotic (Kruis et al. 2004) to pathogenic and deadly (Ron 2006). This family contains many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella* and *Shingella* among others that have been known to proliferate during disease and exacerbate intestinal inflammation in colitis. Representative species from the Enterobacteriaceae family are understood to be some of the first colonizers of the infant gut (Favier et al. 2002).

While the more pernicious species have been studied at length, less is known about how and for what purpose the bacteria reach high levels within the intestinal tract of infants during the first months of life. It has been proposed that the availability of specific milk oligosaccharides select for this family of bacteria. Specifically, elevated levels of the milk oligosaccharide α2,3-sialyllactose (3SL) have been shown to select for higher levels of Enterobacteriaceae during lactation (Weiss and Hennet 2012). Although surprisingly, Enterobacteriaceae cannot feed on 3SL because they lack the proper enzymes for breaking down the units, but rather depend on other intestinal bacteria for that task, such as members of the Bacteroides genus (Huang et al. 2015). While complex oligoasaccharides abound in human breast milk (between 5 and 8 g/liter), only small amounts of oligosaccharides are detectable in cow's milk, with 3SL being a main component (Kunz et al. 2000). High levels of 3SL in cow's milk likely proliferates levels of Enterobacteriaceae, possibly explaining why formula fed infants have been shown to harbor higher levels of *E. coli* and other pathogenic bacteria from the Enterobacteriaceae family (Penders et al. 2005; Palmer 2007).

Not only does cow's milk formula likely contribute to the proliferation of pathogenic bacteria from the Enterobacteriaceae family, but also formula fed infants miss out on specific oligosaccharides that select for other important bacteria, as well as maternal antigens that further hamper the proliferation of such bacteria. Maternal breastmilk sIgA recognize bacterial antigens including Enterobacteriaceae, and coat the bacteria widely (Tsuruta et al. 2010). Coating of bacteria with sIgA hampers their proliferation in the gut, thereby preventing the expansion of colitogenic bacteria (Palm et al. 2014). The importance of maternal sIgA in shaping the gut microbiota has also been demonstrated in newborn mice nursed by mothers unable to transfer sIgA into their milk because of a polymeric Ig receptor defect. Mice fed with antibody-deficient milk presented long-lasting and detrimental changes in their gut microbiota, and increased susceptibility towards colitis (Rogier et al. 2014). The milk proteins lysozyme and lactoferrin also influence the gut microbiota by cleaving cell wall polysaccharides and by chelating iron, respectively. Colostrum is especially rich in lactoferrin (Adamkin 2012), which binds with high affinity to iron, thereby restricting its availability for the growth of pathobionts, such as Enterobacteriaceae (Bullen et al. 1972).

Escherichia coli is thought to be the most prevalent species of the Enterobacteriaceae family that resides in the infant intestinal tract (Park et al. 2005). *E. coli* is a gram-negative, facultative anaerobic, and non-sporulating bacterium, and performs multiple roles within the intestinal tract of infants and adults. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts. The harmless strains are part of

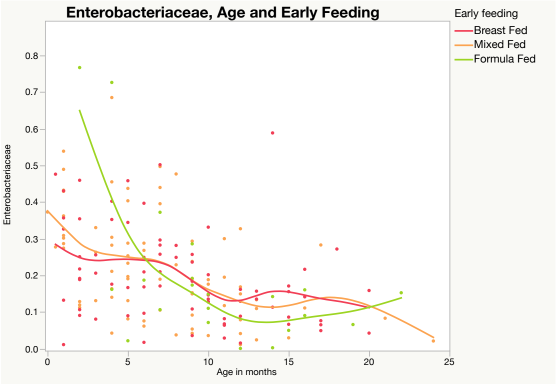

Figure 73: Enterobacteriaceae, age and early feeding.

These bacteria were initially higher in FF infants when compared to BF and MF infants.

the normal flora of the gut, and produce K- and B-complex vitamins in the intestines, which are absorbed by the infant's body as essential nutrients (Bentley and Meganathan 1982). The *E. coli* strain Nissle 1971, stimulates lymphocytes to secret IgA, which protects the intestinal epithelial cells from enteroinvasive bacterial pathogens (Henker et al. 2008). In contrast, *E. coli* strain O157:H7, which is normally not found in healthy infants, and can cause severe damage in the intestinal epithelial cells (Phillips et al. 2000). Breast milk has been shown to inhibit the growth of *E. coli* within the infant digestive tract. Oligosaccharides in particular inhibit the diarrheagenic effect of the toxins produced by *E. coli.*

Study findings

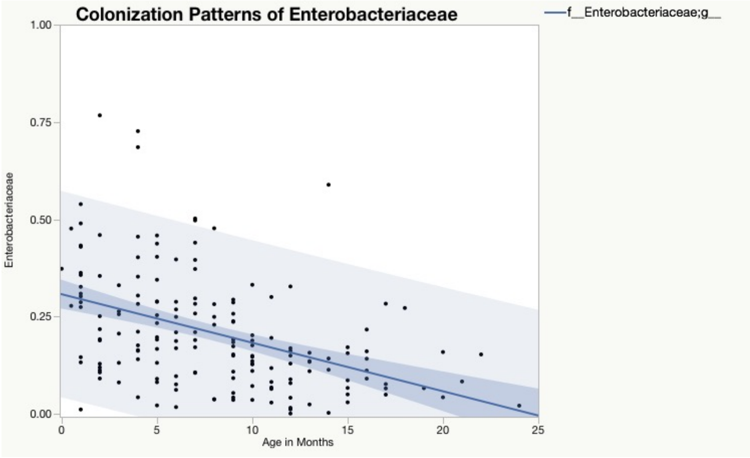

Figure 74: Enterobacteriaceae colonization patterns.

These bacteria are early, abundant colonizers of the infant microbiome in this Salvadoran population. Population levels drop significantly over time, becoming relatively insignificant members of the commensal community by 2 years of age.

Birth-mode was not found to be a significant factor influencing the proliferation of the Enterobacteriaceae family, even after controlling for age. In agreement with the current literature, breastfeeding seemed to highly influence colonization patterns: FF infants harbored nearly double the Enterobacteriaceae compared to BF infants (45.6% and 24.8% respectively) (P=.0004). There was also a significant difference between BF and MF infants (P=.04), suggesting that even a partial diet of cow’s milk formula is enough to encourage the early populating of this family of bacteria (see Figure 73).

Individual subjects and Enterobacteriaceae colonization patterns

Subject #	Age in mo.	Symptomatology	% Relative Abundance	Zwfl	Early Feeding
58	2	Upper-respiratory infection on antibiotics at sampling	77%	.47	MF, cows
42	4	NICU for a month @ birth and not recovered. Multiple doses of antibiotics	73%	-0.85	FF
2	4	Diarrhea the week before sampling	68.5%	1.93	MF
11	14	Allergic to antibiotics, current and prior diarrhea, currently vomiting, refusing food but accepting breastmilk	59%	1.24	MF + comp foods
68	7	Healthy until 6 mo., sick the last month with upper resp. infection. Diarrhea in the last week	50%	-.71	MF + comp foods
66	1	Healthy	1%	.58	BF
18	7	NA	50%	NA	MF
20	8	Series of infections	48%	-1.43	MF/FF

Figure 75: Outliers of Enterobacteriaceae.

These subjects have relative abundances of Enterobacteriaceae that are above or below the reference range. Infants with high levels of the bacterium tend to be symptomatic of infection, and many concurrently have high levels of Enterococcus. Comp foods= complementary feeding. Cows=supplementary powdered bovine milk.

None of the measures of illness detected significant effects on colonization, however the outlier infants who had relative abundances above or below the predictive range (see light grey shadow of Figure 74) tell a different story. Infants above the predictive range had relative abundances of these bacteria between 50-77%. These infants were typically symptomatic of infections (see Figure 75). Conversely, subject 66 had only 1% in her first month, and was a very healthy infant of two comfortable, working parents. Other subjects with low levels of this family of bacteria had recently completed antibiotic rounds that likely reduced levels of this population.

Key words: pathobiont, pathogenic, early colonizer, HMO, 3SL selected for (bovine milk oligosaccharide), co-morbidity with Enterococcus.

Enterococcus

Enterococcus is a large Gram-positive cocci genus of lactic acid bacteria of the Firmicutes phylum. They often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone. Two species, *E. faecalis* (90–95%) and *E. faecium* (5–10%), are common commensal organisms in the intestines of humans, and are relatively resistant to hygienic measures (Kearns et al. 1995). *Enterococci* are facultative anaerobes, as they are capable of cellular respiration in both oxygen-rich and oxygen-poor environments, making them resilient in a wide range of environmental conditions and likely in part contributing to their intrinsic antibiotic resistance (Ryan et al. 2010).

Due to the abundance of oxygen in the neonatal gut, *Enterococcus* is among the first wave of microorganisms detected in the stool of infants (predominantly *E. fecalis* and E. *faecium)* (Penders et al. 2006; Adlerberth and Wold 2009; Vael and Desager 2009). Their expansion leads to a gradual consumption of oxygen to a more reduced environment, which favors the proliferation of obligate anaerobic bacteria, with the dominance of *Bifidobacterium*, *Bacteroides*, and *Clostridium*, followed by *Veillonella*, *Eubacterium*, and *Ruminococcus* species (Penders et al. 2006; Adlerberth and Wold 2009; Vael and Desager 2009). With time, anaerobic species will expand and outnumber facultative bacteria (Penders et al. 2006; Adlerberth and Wold 2009; Vael and Desager 2009), toward an adult-like microbiota profile (Guaraldi and Salvatori 2012). Early colonization by

Enterococcus may be key to early development however, as infants who developed allergies were less often colonized with *Enterococcus* during the first month of life as compared to healthy infants (Björkstén et al. 2001).

There is substantial controversy over the role of *Enterococcus* in health. In clinical settings with immune-compromised patients, *E. faecalis* has been considered an opportunistic pathogen (Pinholt et al. 2014). This species of *Enterococcus* has also been shown to impart beneficial effects to health. A recent in vitro study demonstrated that *E. faecalis* was inhibitory to C. jejuni MB 4185 infection (Robyn et al. 2012). Moreover, *E. faecalis* isolated from a healthy adult showed the highest probiotic activity when compared with over 70 other bacteria, including *lactobacilli* and *bifidobacteria* (Nueno-Palop and Narbad 2011). These contradictory roles suggest the complexity and context dependent interplay between host and bacteria that are likely dynamic over the life-course and vary among individuals.

E. faecalis may also possess the capacity to modulate and attenuate inflammatory responses further to prevent inflammatory diseases such as NEC in infants. An in vivo system revealed anti-inflammatory effects. This finding is consistent with previous study showing that certain isolates of *E. faecalis* have a great protective effect in an experimental colitis model in mice by reducing IL-8 production (Chen et al. 2009).

Theoretically, as one of the first colonizers, the main role of *E. faecalis* may be to suppress early inflammatory responses for the colonization of other microbiota to take residence in the neonatal gut. Exposure to Gram-negative bacteria otherwise can initiate acute inflammation upon exposure, and the presence of *E. faecalis* may help the intestine maintain the immune balance in response to such challenges. The finding that *E. faecalis* performed as good as recognized probiotics indicates their potential to serve as a probiotic. However, the therapeutic effects must be examined thoroughly as *E. faecalis* was also reported as an opportunistic pathogen in hospital infections. To further complicate the matter, one team of researchers demonstrated that when compared with full-term infants, preterm infants showed increased populations of facultative anaerobes such as *Enterococcus* and decreased numbers of anaerobes like *Bifidobacterium*, *Bacteroides*, and *Atopobium* (Berrington et al. 2013; Arboleya et al. 2012), suggesting that Enterococcus should be considered a pathobiont.

Individual subjects and Enterococcus colonization patterns

Subject #	Age in mo.	Symptomatology	% Relative Abundance	Zwfl	Early Feeding
9	1	Alternating diarrhea/ constipation	7.2%	1.19	FF, cows
20	15	Upper resp. infection and antibiotics 1 mo. prior	1.8%	-0.57	MF + comp foods
24	4	Diarrhea the week before sampling, multiple doses of antibiotics	62.5%	.9	FF, cows
58	2	Upper-respiratory infection on antibiotics at sampling	5.3%	.47	MF, cows
68	7	Healthy until 6 mo., sick the last month with upper resp. infection. Diarrhea in the last week	2.1%	-.71	MF + comp foods
70	4	Received vaccinations the day prior to sample collection	3.5%	4.39	MF
5	14	Had bronchitis for a week, was injected with antibiotics the week prior	2.5%	-.8	FF

Table 36: Outliers of Enterococcus.

These subjects have relative abundances of *Enterococcus* that are above the reference range. Infants with high levels of *Enterococcus* tend to be symptomatic of infection, and many of them concurrently have high levels of Enterobacteriaceae. Comp foods= complementary feeding. Cows=supplementary powdered bovine milk.

Study findings

There are extremely low levels of this bacterium in this population (<2%), with a few exceptions. Infant subject 24 at 12 months had 62.5% relative abundance of *Enterococcus*, compared to his peers who averaged < 1% (see Table 36). Clinically he was diagnosed with bronchitis and was taking 7 days of antibiotics (Cefixime). After subject 24 was removed from the dataset, FF infants still had significantly higher relative abundance of these bacteria than do MF (P=.014), and BF (P=.013). Surprisingly, birth mode was not a significant factor in the colonization of these bacteria, although a limitation of the current study in regards to this bacterium, is the lack of data in the days and weeks after birth. It seems likely that *Enterococcus* is an important early colonizer, that successionally contributes to creating an anaerobic niche for future microbiota. Higher than reference ranges may be a marker of a delayed colonization, immature microbiota development, or even antibiotic resistance. The latter is evidenced by higher relative abundances in children that were currently or recently given antibiotics (see Table 36). If species such as *E. faecalis* and *E. faecium* co-exist within a "healthy" microbial community, they must do so at very low numbers after the first month. Multiple infants had both high levels of Enterobacteriaceae and *Enterococcus* simultaneously, indicating dysbiosis and co-morbidity, accompanied by clinical symptoms (i.e. 24, 58, 68). Others had moderately high levels of Enterobacteriaceae with relatively high levels of *Enterococcus* (i.e. 5, 9, 20, and 70).

Key words: Possible probiotic, pathobiont, anti-inflammatory/tolerance inducing in first months, early colonizer, dysbiosis, co-morbidity with Enterobacteriaceae

Faecalibacterium

Faecalibacterium is a Gram-positive bacterium, in the Clostridium leptum group from the Firmicutes phylum. The only known species of the bacteria, *Faecalibacterium prausnitzii*, is an important inhabitant in the human intestinal tract. In healthy adults, *F. prausnitzii* represents more than 5% of the bacteria in the intestine, making it one of the most common gut bacteria (Miquel et al. 2013). *F. prausnitzii* species are a major representative of Firmicutes phylum, Clostridium class, Ruminococcaceae family. These bacterium are non-spore forming, Gram-positive and extremely oxygen-sensitive, making it difficult to cultivate even in anaerobic conditions (Duncan et al. 2002). Despite being extremely oxygen sensitive, *F. prausnitzii* attaches itself to the gut mucosa where oxygen diffuses from epithelial cells. Recent research helps explain this paradox through microbial fuel cell experiments and flavin as prebiotic source (Khan et al. 2012). The results showed that *F. prausnitzii* employs an extracellular electron shuttle of flavins and thiols to transfer electrons to oxygen (ibid). Both compounds are present in the healthy human gut, allowing this bacterium to survive. Future research will assess possible implications for the treatment of patients with inflammatory gut conditions with flavin or antioxidant rich diets as prebiotic sources.

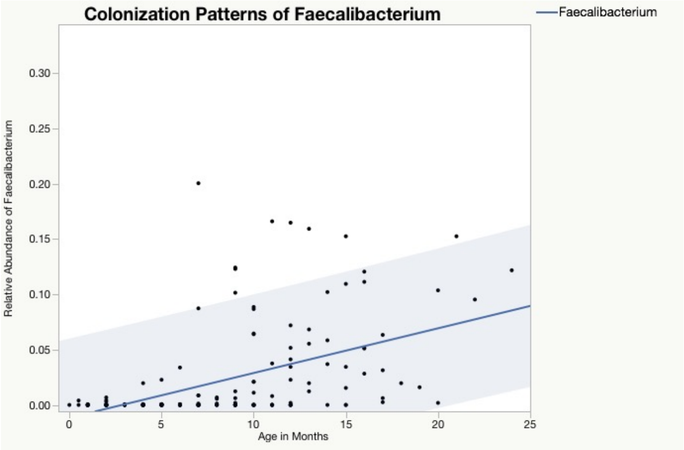

Figure 76: *Faecalibacterium* colonization patterns.

These bacteria are later colonizers of the infant microbiome in this Salvadoran population. Population levels increase steadily after birth in some infants, although others maintain levels <5% throughout the first 2 years. This bacterium is a keystone species and demarks a “healthy” infant microbiome as well as average growth phenotypes.

The major end products

of glucose fermentation by *F. prausnitzii* strains are formate, small amounts of D-lactate (L-lactate being undetectable) and substantial quantities of butyrate (>10 mM butyrate in vitro) (Duncan et al. 2002; Duncan et al. 2004) and other short-chain fatty acids through the fermentation of dietary fiber. These activities are critical for the health of the microbiome, such that this species is commonly referred to as a "keystone species." Recently, culture medium supplemented with flavins and cysteine or glutathione was shown to support growth of *F. prausnitzii* under micro-aerobic conditions (Khan et al. 2012).

In fact, low or depleted levels of *F. prausnitzii* in the intestines have been associated with an number of diseases, including Crohn's Disease (Sokol et al. 2008), appendicitis (Swidsinski et al. 2011), and obesity (Hippe et al. 2016). Studies have demonstrated that *F. prausnitzii* may have pronounced anti-inflammatory effects. Sokol et al. (2008) studied the effect of *F. prausnitzii* in peripheral blood mononuclear cells and the results suggest that *F. prausnitzii* may induce an increased secretion of an anti-inflammatory cytokine interleukin 10, and a decreased secretion of pro-inflammatory cytokines like interleukin 12 in vitro. Sokol and colleagues (2008) have also studied the effect of *F. prausnitzii* in the prevention of colitis in mice. *F. prausnitzii* was found to induce an increased interleukin 10 and a decreased interleukin 12 and tumor necrosis factor –α production in vivo, and the daily administration of *F. prausnitzii* led to marked attenuation of colitis. These findings suggest that *F. prausnitzii* may have a capability to suppress inflammation, and it is hypothesized that this is due to metabolite(s) secreted by F. prausnitzii. The identification of the active molecule(s) involved in this anti-inflammatory effect is the subject of research

(Sokol et al. 2008).

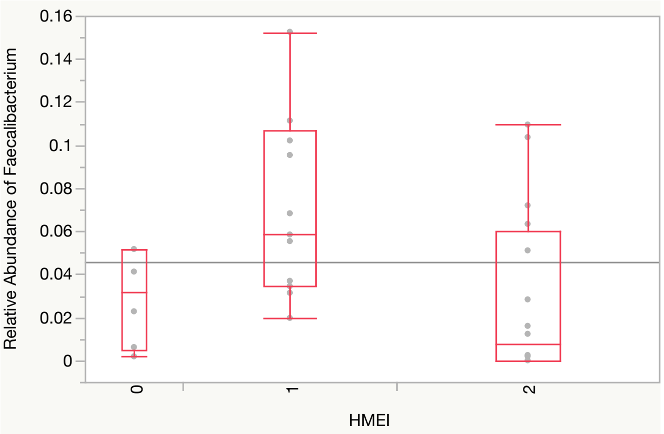

Figure 78: *Faecalibacterium* and HMEI exposures at 12+ months.

The middle HMEI group (1) have significantly higher relative abundance of *Faecalibacterium* than infants in the high HMEI group (2) (P=.01). Although not significant, the low HMEI group (0) seems to also harbor lower relative abundance compared to the middle group.

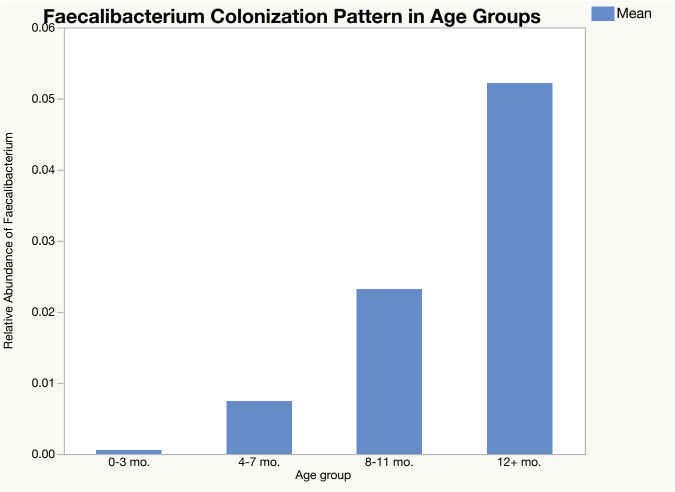

Figure 77: Faecalibacterium colonization in age groups.

This bacterium was detectable in this population at 0-3 mo. and continued to colonize infants, reaching the peak average abundance by 12+ mo.

Although *F. prausnitzii* is a keystone species in the adult gut, known to have anti-inflammatory effects, the development in infants and the functional activities of the bacterium is understudied. One study found that Malawian and Bangladeshi infants were colonized with *F. prausnitzii* only after 12 months and reaching the highest mean relative abundance at 19 months (Blanton et al. 2016). Another study found that most Japanese infants had undetectable levels at 0-1 years, but the count reached a level comparable to adults in 2-5-year-old infants (Koga et al. 2016).

Study findings

Individual study participants began to acquire detectable levels of these bacteria within the first three months of life, showing earlier colonization patterns than previously found in other populations (Blanton et al. 2016; Koga et al. 2016) (see Figure 76). The current study population displayed a relatively even gradient colonization pattern, reaching the highest relative abundance at 21 months (see Figure 76 and Figure 77). Although birth-mode and early feeding were not significantly related to levels of the bacterium in study infants, BF infants consistently had higher levels of the bacterium (see). Moreover, ten infants were outliers with relative abundance above the predictive range, and will be described below and in Table 37.

Individual subjects and Faecalibacterium colonization patterns (12+ mo.)

Subject #	Age in mo.	Symptomatology/ Field Notes	% Relative Abundance	Zwfl	Early Feeding
13	21	Unknown infection, on antibiotics	15.3%	-0.57	MF
22	7	Unknown GI infection, on antibiotics, high environmental exposure (i.e animals)	20%	NA	BF + comp foods
23	9	Sick with diarrhea but not antibiotics, high environmental exposure (siblings, animals, household)	12%	-0.79	BF + comp foods
32	11	Infant has a cold, almost always has upper resp. infections, antibiotics	17%	-1.44	BF + comp foods
40	9	Child recently treated with antibiotics for bronchitis, high environmental exposure (siblings, animals, household)	12.4%	0.79	MF + comp foods
42	7	NICU for a month @ birth and not recovered. Multiple doses of antibiotics	8.7%	-1.9	FF + comp foods
45	5	High environmental exposure (i.e. large family, 6 dogs, cats, ducks, chickens)	16.5%	-.8	BF
48	13	GI infection 2 weeks prior, antibiotics, high environmental exposure (large family, animals, farm)	16.9%	0.02	BF + comp foods
53	9	History of Gi infections and antibiotics (but seemingly healthy at interview), mother is chronically unwell (undiagnosed condition)	10.2%	-1.38	BF + comp foods
67	15	Obese child, had upper resp. infection, 10 days antibiotics 1 week prior. High environmental exposure (siblings, food)	15.2%	2.59	BF + comp foods

Table 37: Outliers of *Faecalibacterium*.

These individuals harbor levels of this bacterium that are above the reference range. Infants with high levels of this bacterium tend to be BF, high environmental exposures, high rates of antibiotics, and symptomatic of infection. Comp foods= complementary feeding.

Interesting associations were found between *Faecalibacterium* and the HMEI scale. Specifically, infants that were in the middle HMEI group had significantly higher levels of the bacterium than infants of the high HMEI group, suggesting that a moderate degree of horizontal exposure is important for the acquisition of this bacterium. While not significantly different, the low HMEI group also appeared to have lower average levels of relative abundance compared to middle HMEI infants. Figure 78 shows a resemblance to associations between Zwfl and HMEI scores also at 12+ mo., and possibly underscores the importance of this bacterium in growth at this age. In fact, this bacterium was highly associated with healthy growth outcomes: AGP infants at 0-3 months had positive correlations with Zwfl, and again at 12+ months. Functional activities of *Faecalibacterium* including increased carbohydrate fermentation, energy extraction and butyrate production may be responsible for this healthy growth augmentation. Additionally, the OGP group had the highest mean levels of this bacterium, suggesting that it may be a key player in weight gain and growth support for this population.

However, upon examination of infants who harbored levels of this bacterium above the predictive range (Table 37), it seems that there may be a specific levels that supports growth and positive health outcomes. This may be particularly true of younger infants, when successional development is key for the proper maturation of the intestinal tract, immune system and microbiome more generally.

General characteristics of these outliers pointed to surprising trends: 7/10 of the infants were breastfed, most of whom were supplemented with complementary foods because of age. Also, 7/10 of these infants were currently on or had recently taken at one or more doses of antibiotics (<2 weeks). Another pattern among 6/10 participants, was the high degree of environmental exposure. These infants experienced high levels of interactions with older siblings, exposure to animals in the household, received an early introduction of complementary foods, among other environmental exposures.

Subject 40 at 9 months was being cared for by her 6-year-old sister and 4-year-old brother while their parents worked. Upon multiple visits, we found the infant in long-soiled diapers and tied to a chair while the older sister could better feed and care for her. The older sister was not strong enough to lift the baby onto the bed, and thus the baby spent the day on the floor or in the chair. Upon arrival during one visit, I encountered the 4-year-old defecating in front of the house. This perhaps was because the only access to an outhouse was through to a neighbor's yard, or because the four-year-old was supervised by his six-year-old sister. There was no running water in this household. It is unsurprising that the infant suffered from common GI and upper-respiratory infections, and had been administered multiple doses of antibiotics (with unknown adherence rates).

The mother of subject 22 reported that she began feeding the infant foods "to taste" just before four months of age. The infant immediately became ill and had diarrhea for 10 days. The mother took the infant to a private clinic and was treated with multiple doses of antibiotics. She said he hadn't been well since (he was currently on antibiotics when the sample was taken). This household also had many animals on their grounds, including dogs, chickens, ducks and parrots, providing a lot of horizontal exposure to microbes.

Subject 23 was 9 mo. with extremely high rates of exposure to siblings, animals, and a generally messy household. During one visit, the child pooped his pants, although because he wasn't put in diapers, the mother took him to the kitchen sink to wash him. He sat there waiting for his bath and picked up and ate piece of carrot that was next to him among the dirty dishes, soiled pants, old food and trash. His mother said she had been busy making pupusas (a typical Salvadoran lunch food), and she hadn't had time to bathe him that day.

Although the focus of this study on the early months of development inhibits a clear view of factors that affect the colonization of this bacterium in the second year, it seems likely that this microbe is acquired through environmental exposures. Future research should explore childhood ecologies from ages 9 months to 3 years to better understand the sources, exposures, and development of these bacteria. The early acquisition of this bacterium may in part be explained by the early introduction of complementary foods in this population. It also seems that while it may be a keystone species within the adult microbiome, the timing of colonization and relative abundance levels are key to its beneficial activities within infants.

Key words: Keystone species, healthy biomarker anti-inflammatory, >12 months colonization, associated with growth 0-12months in AGP infants.

Lachnospiraceae (family)

The Lachnospiraceae family consists of 24 named genera and several unclassified strains (Sayers et al. 2010), including *Ruminococcus*, *Blautia*, *Dorea*, and *Lachnoanaerobaculum* as well as a number of *incertae sedis* strains (Sayers et al. 2009) sharing a high degree of similarity among their 16S rDNA sequences (Dworkin 2006). All known family members are strictly anaerobic (Dworkin & Falkow 2006), reside mainly within the digestive tracts of mammals (Bryant

1986; Carlier et al. 2004; Downes et al. 2002; Moon et al. 2008) and are thought to be primarily non-spore-forming (Dworkin & Falkow 2006). Several members play key roles within the human GI microbiome, demonstrated by their inclusion in an artificial bacterial community that has been used to repopulate a gut microbiome and remedy *Clostridium difficile* infections (Petrof et al. 2013).

Conversely, blooms of Lachnospiraceae in early life may be linked with obesity (Cho et al. 2012), most likely due to their short chain fatty acid (SCFA) production (Duncan et al. 2002). Lachnospiraceae constitutes one of the major taxonomic groups of the human gut microbiota that degrade complex polysaccharides to SCFAs, including acetate, butyrate, and propionate, that can be used for energy by the host (Biddle et al. 2013). One study showed that early-life sub-therapeutic antibiotic treatment lead to shifts in taxonomic composition, primarily blooms of Lachnospiraceae. These researchers suggested that these bacterial family may have roles in the development of an obesogenic metabolic phenotype (Cho et al. 2012). However, despite their apparent importance, little is known about their presence and possible roles played by these bacteria in the early life of humans (Sagheddu et al. 2016).

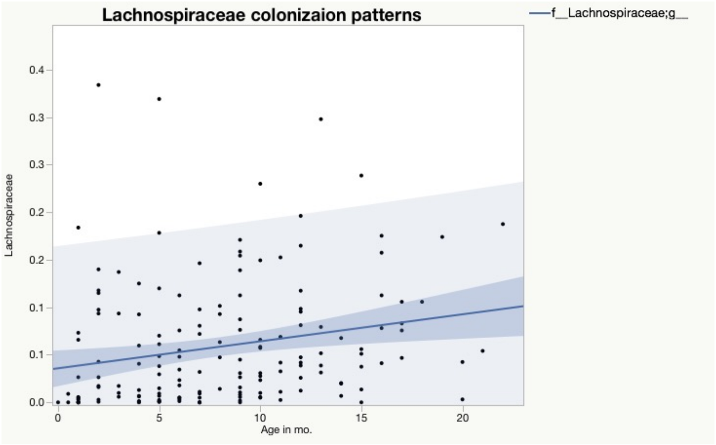

Figure 79: Lachnospiraceae colonization patterns.

These bacteria increase in relative abundance over time. 8 infants had higher than predicted levels.

Study findings

Lachnospiraceae colonized the infant microbiome at a steady pace beginning after birth and continuing through the end of the study (see Figure 79). Birth mode was not significantly related to levels of these bacteria. In the current study, FF infants had significantly higher levels of Lachnospiraceae (F=18.15, P=.0001) than exclusively BF infants and MF infants (P= .01). MF infants had significantly higher Lachnospiraceae than BF infants (P=.03) (see Figure 80). Moreover, currently breastfeeding babies had significantly lower relative abundance of the bacteria than FF infants (after controlling for age), suggesting that breastmilk is protective from early blooms of these bacteria. Higher levels of these bacteria were not significantly related to growth markers in this population, although this does not rule out the risk that early blooms will influence weight gain in early childhood and beyond.

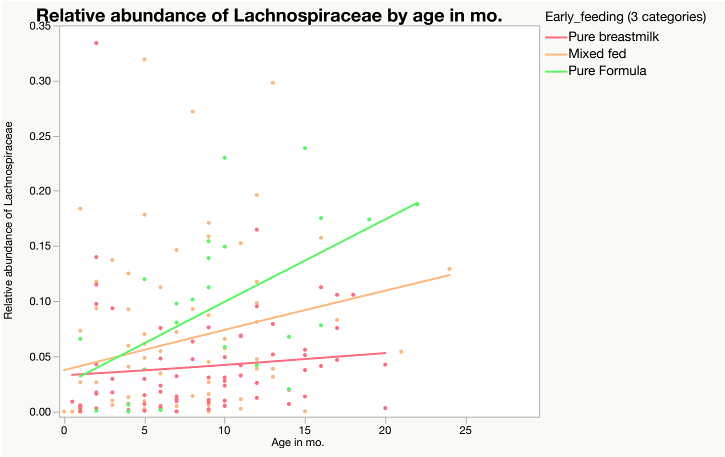

Figure 80: Patterns of Lachnospiraceae colonization over time by feeding method.

Breastfed infants show a slower, more stable increase in bacteria from this family. Formula fed infants show the most dramatic increases over time.

Another interesting finding was, as α-diversity increased, the relative abundance of Lachnospiraceae also increased significantly (F=18.15, P=.0001) Moreover, mixed and formula fed infants showed a stronger trend where the more diversity they have, the more likely they are to acquire Lachnospiraceae. This may be a key bacteria in understanding why alpha diversity may not be optimal during the early months. Finally, Infants who have not received antibiotics have significantly lower levels of Lachnospiraceae (P=.02). Thus early feeding practices and overuse of antibiotics seem to be major factors in the levels of these bacteria. Given that high early populations are associated with obesity, this may be a key bacteria in determining and altering obesogenic trajectories in infants and children.

Key words: Pathobiont, early blooms associated with obesity, SCFA production,

Lactobacillus

Lactobacillus are Gram-positive rods that can be found throughout the digestive tract, but are predominantly present in the large intestine (Tannock et al. 2013). Birth mode and early feeding are likely important means of infant colonization by *Lactobacilli* (Fanaro et al. 2003), and are second only to *Bifidobacteria* in dominating the microbiota of breast-fed infants. One study suggested that the mode of delivery, but not the method of feeding, had a significant influence on early colonization (Hall et al. 1990). Previous treatment with antibiotics

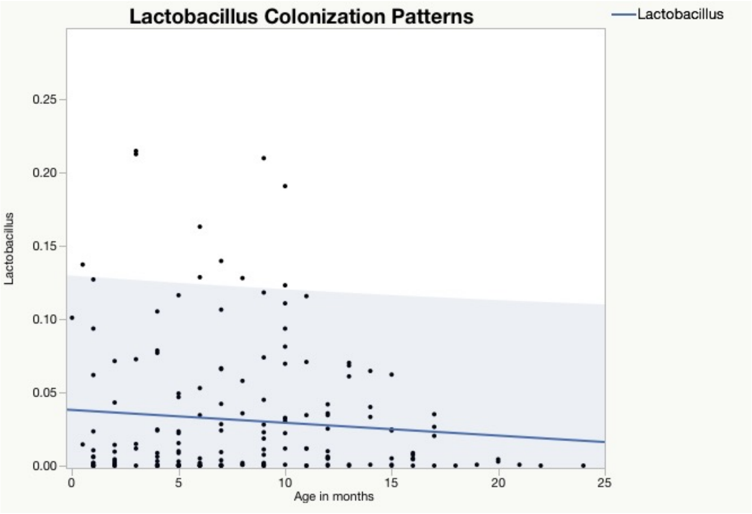

Figure 81: Lactobacillus decrease over time.

There are low levels of this bacterium in this Salvadoran population.

and being nursed in an incubator were also significantly associated with a lower rate of early colonization with *lactobacilli* (Hall et al. 1990)

The most common species of *Lactobacillus* found in infants is *Lactobacillus acidophilus* (Wall et al. 2008). *Lactobacilli* contribute to digestion, stimulate the immune system, and inhibit the growth of pathogens (Haarman and Knol 2006). They live in habitats rich in carbohydrates, such as an infant's digestive tract. *Lactobacilli*, a member of the lactic acid bacteria group, break down sugars, mainly lactose, into lactic acid using the enzyme β-galactosidase. Sugar metabolism provides nutrients and energy for its growth and survival (Parracho et al. 2005; Tannock et al. 2013). The accumulation of lactic acid lowers the environmental pH, which inhibits the growth of pathogenic bacteria, such as *Helicobacter pylori*. *Lactobacilli* can regulate their enzymatic activity to achieve a more suitable or optimal living condition. They can also inhibit growth of other bacteria by competing with them for nutrients and adhesion sites on the epithelial lining of the intestinal wall (Parracho et al. 2005). commonly used as probiotics, supplements containing bacteria

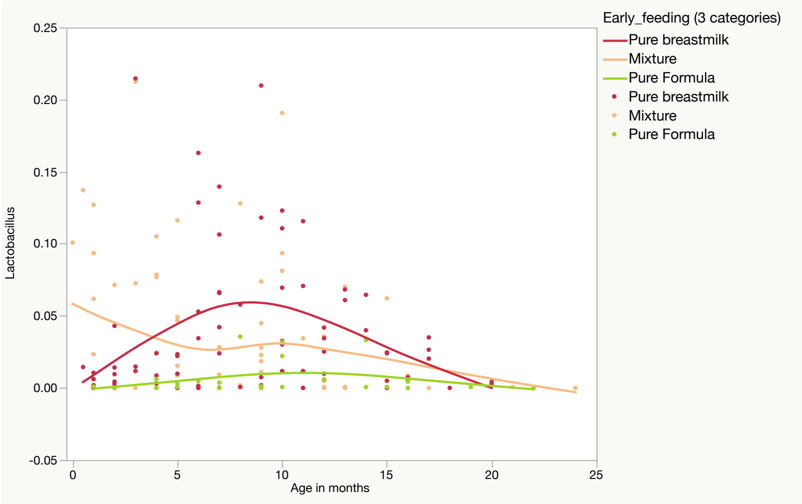

Figure 82: Lactobacillus colonization patterns by early feeding practice.

Breastfed infants increase relative whereas mixed fed abundance of Lactobacillus over time, peaking in the 10th month and whereas mixed fed decreasing thereafter, whereas mixed fed infants peak earlier and steadily decrease in abundance. Formula Fed infants maintain low levels of Lactobacillus throughout this early developmental stage.

that are beneficial to humans (Wall et al. 2008; Tannock et al. 2013). Babies who start out with fewer *Lactobacilli* are more likely to develop allergies later in life, and giving *Lactobacillus* supplements to infants at high risk for allergies and asthma may prevent those conditions from developing (Forsberg 2016). Conversely, antibiotics administered to pregnant rats, have been shown to reduce the relative abundance of *Lactobacillus* in offspring (Khan et al. 2016). Use of category B antibiotics (azithromycin, amoxicillin, and cefaclor) during pregnancy was shown to increase the fecal relative abundance of Proteobacteria and *Enterobacter*, while reducing the relative abundance of Firmicutes and *Lactobacillus* (ibid).

Low doses of antibiotics were associated with diminished reserves of the bacterium in offspring (Cox et al. 2014).

Study findings

Extremely low levels of *Lactobacillus* were found in this study population. Like the experimental studies previously mentioned, this may in part be explained by the high levels of prophylactic antibiotic treatment of pregnant mothers in this population. Unfortunately, data were not collected on perinatal maternal

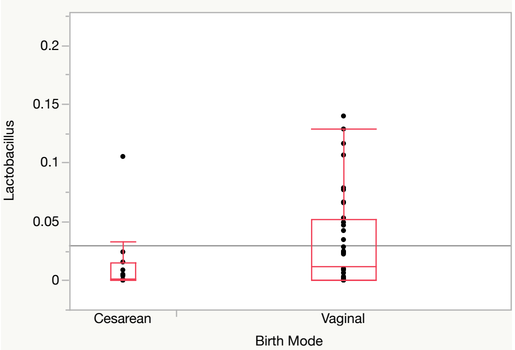

Figure 83: Birth mode and Lactobacillus colonization.

Birth mode significantly affects colonization patterns of Lactobacillus. Infants born via Cesarean section have significantly lower levels of the bacteria than do vaginally birthed infants (F=5.4, P=.02).

antibiotic administration, nor on prenatal maternal vaginal microbiomes.

Although only found in low abundance, *Lactobacillus* colonizes infants early in this population, with decreasing relative counts after 10 months (see Figure 81). Vaginally delivered infants harbored significantly higher levels of *Lactobacillus* than cesarean delivered infants (F=5.4, P=.02) (see Figure 83). Early feeding was also a significant factor in the colonization of this bacterium (see Figure 82). Infants who were breastfed and those who were mixed-fed had significantly higher relative abundance of *Lactobacillus* than infants who were formula fed (P=.01, P=.04 respectively). Interestingly, during the first 3 months of life, VDBF infants had the highest averages of this bacterium (2.62%), whereas CDBF had only .71%, and VDFF only .05%, suggesting that early bacteria seeded through vaginal delivery may affect colonization only when enhanced by breastmilk. In part this may be explained by a synergism amongst microbiota. For example, in this Salvadoran population, *Bifidobacterium* likely populated the infant gut through both birth-mode and through breastmilk. *Bifidobacterium* as a genus is known to create an acidic barrier against pathogenic bacteria, which also creates a favorable environment for *Lactobacillus*. Thus, *Lactobacillus* and other vaginally associated bacteria that were seeded at birth may flourish only in the presence of other bacteria. It is also possible that mothers in this study population harbor fewer *Lactobacillus* in their vaginal canal, making birth-mode a less significant factor the colonization of this bacterium, as well as a less dominant bacterium in general when compared to other populations (Dominguez-Bello et al. 2010). These hypotheses are in part validated by assessing infants who have higher than the predictive range of *Lactobacillus*: 9/11 of the infants were VD, 7/11 were BF, and importantly none were FF. All outlier infants were generally healthy, and none showed adverse effects to high levels of the bacterium.

Although *Lactobacillus* has never been directly associated with early ecology, middle HMEI infants harbor significantly higher levels of Lactobacillus (F=4.9, P=.009) than infants of the low (P=.028) or high HMEI group (P=.032). This suggests that early infant ecologies may influence or facilitate levels of Lactobacillus. Middle HMEI infants tend to have moderate exposure to environments, and be ill less often than lower HMEI infants. These infants tend to have the most stable microbiomes, the best growth outcomes and it is likely in part due to the higher levels of *Lactobacillus* in their gut microbiomes. Although it was only found at low levels in the general population, *Lactobacillus* was positively correlated with all four growth measures, including Zwei, Zwfl, Zlen and Zbmi. Interestingly, underweight infants had the lowest levels of *Lactobacilli* at all age groups, highlighting the importance of this bacterium to infant growth and health. Antibiotics did not significantly affect levels of this bacterium.

Key words: probiotic, lowers pH balance, pathogen inhibitor, sensitive to early feeding

Megasphaera

Little is known about this family of bacteria. It has recently been discovered that they bacteria produce important metabolites like short chain fatty acids (butyrate, acetate, formate, and caproate), and vitamins and essential amino acids (Shetty et al. 2013). For these reasons, *Megasphaera elsdenii* is suggested to be an ecologically important rumen bacterium whose genome has only recently been sequenced (Marx et al. 2011). This bacterium metabolizes DL-lactate principally to propionate and acetate (Counotte et al. 1981; Elsden et al. 1956; Marounek et al. 1989). Recently lacate-limited cultures (glucose heavy diets) have been shown to produce in a mixture of acetate and butyrate (Prabhu et al. 2012). The ability of this bacterium to produce

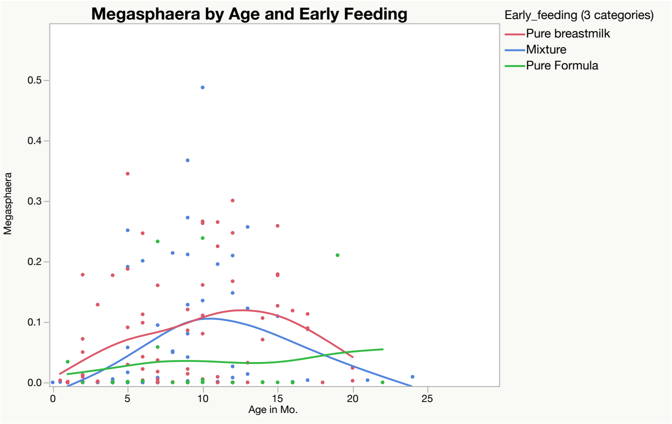

Figure 84: Megasphaera by age and early feeding.

Relative abundance of Megasphaera in early feeding groups. Infants who were FF had significantly lower levels of Megasphaera than BF and MF infants.

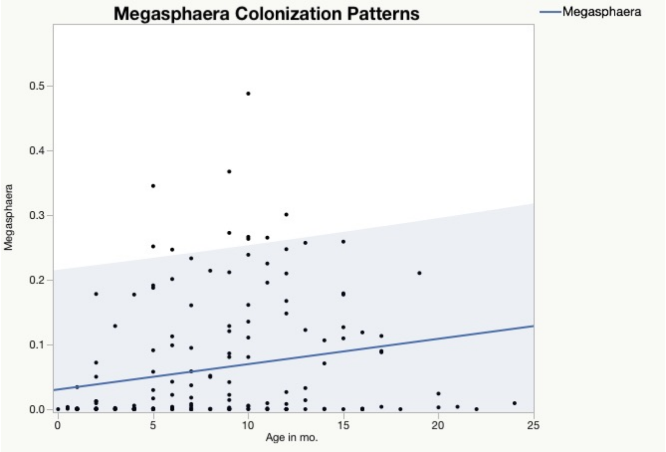

Figure 85: Megasphaera colonization patterns.

These bacteria steadily increase in abundance in this population, peaking around the 8-12th month when they are also significantly (postitively) associated with growth. Infants who were FF had significantly lower levels of Megasphaera than BF and MF infants.

important metabolites suggests a potentially healthy role (for the host) in the gut microbiome.

Study findings

Birth mode was not found to be a significant factor in the colonization of *Megasphaera*. After controlling for age, early feeding was a significant factor: BF infants have significantly higher relative abundance of the bacteria than FF infants (P=.0013) (Figure 84). Nine infants were found to harbor higher levels of the bacteria than the predictive range (Figure 85): of these infants, 7 were BF and 2 were MF (none FF), and only 2/9 were CD. These findings indicate that in this population, birth mode and early feeding matter for the increased population of this bacteria. Antibiotic consumption ranged from 0-6 doses, suggesting that this bacterium is not particularly sensitive. *Megasphaera* became positively correlated with Zwfl around 8-12+ months, which was also the peak of its colonization. Further research will need to be conducted on the functional significance of this bacterium, its colonization patterns and its associations with growth.

Key words: SCFA producer, metabolizes lactate, early feeding

Prevotella

Prevotella is a genus of Gram-negative bacteria, with a relatively high species and even strain diversity (Ruth E. Ley 2016). *Prevotella* spp. are members of the oral, vaginal and gut microbiomes, and have been recovered from anaerobic infections of the respiratory tract (Kedzia et al. 2003), as well as being associated with periodontal disease and periodontal abscesses (Tanaka et al. 2008). Culture collections now include ~40 different *Prevotella* species, most of them oral isolates, and three of which are found in the gut (*P. copri* is generally the more abundant). In a comparison of the genomes of 39 *Prevotella* species, Accetto and Avguštin (2015) observed that *P. copri* DSM 18205 is deficient in the ability to degrade host glycans and its gene repertoire is more geared towards plant glycan degradation, which fits with its niche as a gut fermenter. Based on comparisons between the genomes of 28 *Prevotella* spp., Gupta *et al.* (2015) suggested that individual members of *Prevotella* spp. adapt to niches within the human body by modulating their gene repertoires with extensive gene acquisition and loss.

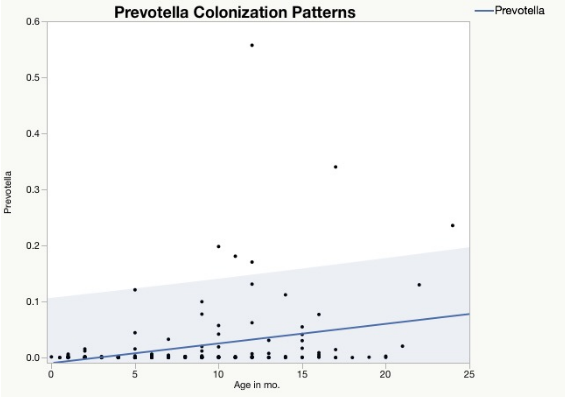

Figure 86: Prevotella colonization increases steadily with age.

Of the members of the Bacteroidetes, two genera dominate — *Bacteroides* and *Prevotella*. Multiple studies have shown an apparent trade-off in numerical dominance between these two bacteria within the gut microbiome. While the majority of Westerners harbor an abundance of *Bacteroides*, *Prevotella* is more common in non-Westerners who consume a plant-rich (high carb and fiber) diet (Martínez et al. 2015; De Filippo et al. 2010) and has been linked to vegetarianism in Western populations (Wu et al. 2011). One study found that *Prevotella* made up 53% of the gut bacteria among children in Burkina Faso, and yet were absent in age-matched European children (De Filippo et al. 2010). Associations with plant-based diets have suggested that *Prevotella* is a beneficial microbe. However, *Prevotella* in the gut has also been linked with inflammatory conditions (Scher et al. 2013; Dillon et al. 2016b), showing this genus to be a context-dependent pathobiont.

In human populations, however, strain variation compounds the uncertainty for predicting how *Prevotella* will function in any given gut ecosystem. To more accurately predict its function will require a finer-grained understanding of these species' genetic potential, their ecology, their ontogeny, their interactions with other microbes present and with their host. A deeper understanding of strain-level genome content on a per-individual basis, obtained through metagenomic techniques, will inform attempts to modulate levels of these bacteria therapeutically either for improving metabolism or reducing risk of inflammation in susceptible hosts.

Study findings

Abundances of *Prevotella* increase steadily after the fifth month of life, and do not seem to be affected by birth mode or early feeding practices (see Figure 86). Much of the current literature links prevalence of *Prevotella* to diet, although in the current study it seems more influenced by social and ecological conditions including the number of different types of animals that inhabit the household and levels of sanitation in the household. After controlling for age, infants in the low-HMEI group have significantly higher relative abundances of *Prevotella* than infants in the medium or high HMEI groups (P=.03) (see Figure 88). Interestingly, SES level was not a predictor of colonization by this bacterium. The number of individuals making up an infant care network was positively associated with higher levels of *Prevotella* (F=4.9, P=.008). Although the differences are not significant, it does seem that there is a trend in the number of different types of animals kept in a household (Figure 87), and

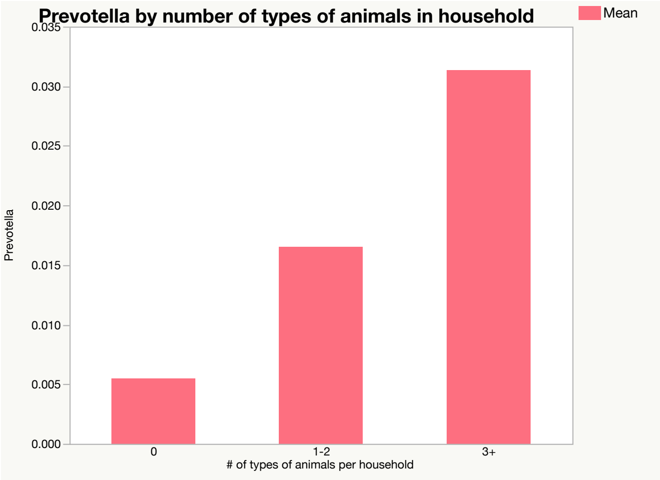

Figure 87: Prevotella colonization patterns with animals in house.

Infants who live in households with 0 animals have the lowest relative abundance of Prevotella.

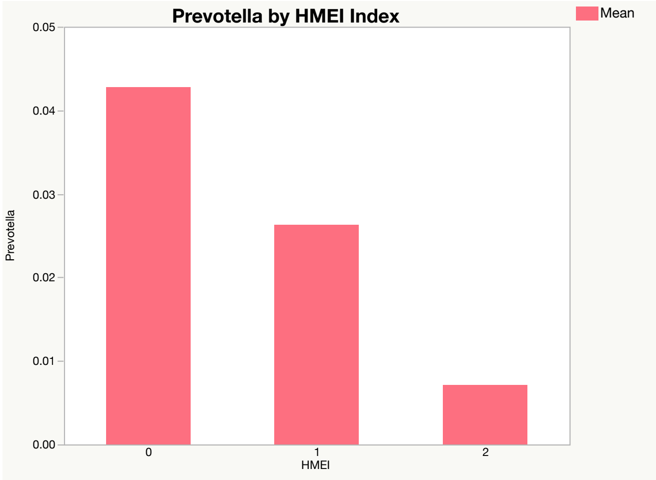

Figure 88: Prevotella by HEMI groups.

Infants in the low HMEI population have higher relative abundance of Prevotella than infants in the other to HMEI goups.

household population density. These findings together strongly suggest that Prevotella is a bacterium that is horizontally acquired through human ecology, and possibly not primarily through diet. Like Blanton et al. (2016) and Subramanian et al. (2014) who found that *Prevotella* was associated with healthy growth, I found this association in the general population but also observed that specifically in the AGP infants, *Prevotella* was positively correlated with Zwfl scores at 12+ months.

Of the four unique outliers, half were CD, and all infants were either BF or MF (4/6 and 2/6 respectively) indicating that early feeding may be more affected by the high colonization of this bacterium. These infants had high levels of microbial exposure, whether to animals, environmental or living conditions, high household population density. Finally, 5/6 of the infants had a history of high antibiotic consumption, and the 6th infant was sick often but his mother was unconcerned about taking him to doctor's visits. Together these data are pointing to environmental exposures as the most important colonization factors, rather than diet alone as many studies indicate.

Key words: *Bacteroides/Prevotella* tradeoff, pathobiont, plant glycan degradation, linked to vegetarian diet, and HMEI scale

Roseburia

Roseburia is a Gram-positive anaerobic bacterium that inhabits the human intestinal tract, and a member of the phylum firmicutes. Increased abundance of *Roseburia* is associated with weight loss and reduced glucose intolerance (Ryan et al. 2010). These beneficial bacteria produce SCFAs including butyrate which may act as an anti-inflammatory and even prevent colon cancer. For this reason, these microbes are thought to be markers of a healthy gut, and are depleted in patients with liver disease and irritable bowel disease.

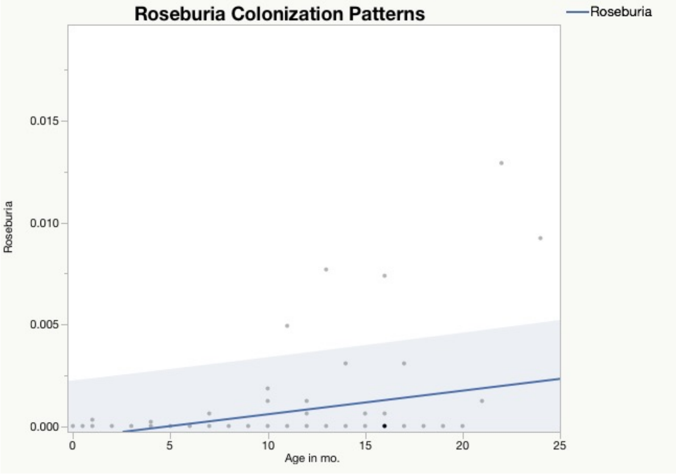

Figure 89: Roseburia colonization patterns.

Infants acquire this bacterium in higher abundance beginning in their 10th month. Five samples, and four unique infants were above that predictive range for this bacterium.

Studies show that infants do not acquire these bacteria until after *Lactobacillus* and *Enterobacteriaceae* populations decrease between 9 and 18 months. *Roseburia* and other butyrate-producing taxa increase from age 9-36 months (Bergström et al. 2014). This is in accordance with previous findings seen at cessation of breastfeeding and introduction of formula feeding and/or cow's milk (Adlerberth and Wold 2009; Fallani et al. 2011; Roger et al. 2010; Edwards and Parrett 2002).

Study findings

Infants who harbored this bacterium, acquired *Roseburia* after 10 months of age, although it was a relatively rare bacterium in this population (see Figure 89). Early care practices were not found to be significant for the acquisition of these bacteria in infants, and is likely beyond the scope of this paper but important for future research to consider.

There were only four infants who harbored higher than reference range levels of this bacterium, although two of these infants had high levels at two distinct ages. Two infants were VD and two were CD. Three infants were exclusively BF. Further research should examine the early care and environmental factors that influence the development of *Roseburia* in infants.

Key words: anti-inflammatory, SCFA, biomarker of healthy microbiome in adults.

Rothia

Species of this genus are part of the normal flora of the human oropharynx and upper respiratory tract (Trivedi and Malhotra 2015). They are aerobic or facultatively anaerobic non-motile, non-spore-forming, Gram-positive coccobacilli that can form filamentous branches (ibid). *Rothia* spp. are commonly associated with dental caries and periodontal disease (ibid). Invasive disease does occur, predominantly in immunocompromised hosts, but has rarely been reported in healthy hosts. The clinical syndromes associated with *Rothia* infection have included bacteremia (Vaccher et al. 2007), endocarditis

(Bruminhent et al. 2013), bone and joint infections (Trivedi and Malhotra 2015), and pneumonia (Cho et al. 2013) among other infections.

One group of researchers found that three-month-old infants who harbored low levels of four types of bacteria — in the genera *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* — were more likely to be diagnosed with asthma by age three. Of the 319 children, 22 fell into this high-risk group, eight of whom were later diagnosed with asthma (Arrieta et al. 2015). The results point to a "critical window" in the first 100 days of life in which disruptions in the healthy development of the gut microbiome can lead to asthma (ibid).

Study findings

The sequencing did not account for this genus.

Key words: Pathobiont, possibly protective against asthma, not sequenced

Ruminococcus

Ruminococcus is a genus of bacteria from the class Clostridia. These bacteria are anaerobic, Gram-positive microbes. Members of this genus are typically found in abundance in the human gut microbiome. *Ruminococcus* is also present in the stool samples of neonates and infants (Favier et al. 2002; Magne et al. 2006; Coppa et al. 2011). The presence the *R. gnavus* seems to be not strictly dependent on the delivery and feeding mode, although likely it influenced by these early factors (Sagheddu et al. 2016). The presence of this bacterium was also confirmed in babies fed soy milk (Piacentini et al. 2010) and those fed goat milk (Tannock et al. 2013). Results of this latter study suggested a relevant presence of members of the species of *R. gnavus* in babies fed breastmilk or goat-milk formula compared with babies fed cow milk formula, while *Bifidobacteriaceae* were abundant in the microbiota of infants in all three groups.

These studies indicate that the presence of *R. gnavus* is predominant in the infant gut at levels as high as *Bifidobacteria* and is not dependent on the type of delivery and feeding. This finding is particularly relevant because *Ruminococci* and *Bifidobacteria* share metabolic pathways involved in complex sugar degradation (Cervera-Tison et al. 2012) and in the degradation of mucin (Crost et al. 2013). Thus, the presence of high levels of *R. gnavus* is a notable finding and supports the suggestions that the release of sugars by the mucin degradation might be important for succession by other bacteria (O'Toole and Claesson 2010). Further investigations are required to ascertain whether the high level of *R. gnavus* has functional consequences. Blanton et al. suggested a putative role of *R. gnavus* in ameliorating growth and metabolic abnormalities in animals receiving fecal transplantation from malnourished babies aged 6–18 months (Blanton et al. 2016). Their results support the hypothesis that *R. gnavus* plays a role in promoting protein synthesis and lean body mass formation instead of amino acid oxidation. The results obtained in a murine model indicate that *R. gnavus* may help in preventing malnutrition and clearly support the relevant role of this organism in the assessment of infant gut microbiota (Sagheddu et al. 2016).

Study findings

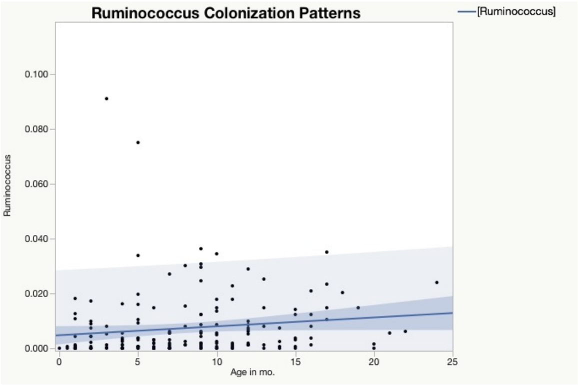

Figure 90: Ruminococcus colonization patterns.

Levels of *Ruminococcus* increase steadily over time, although it is an early colonizer.

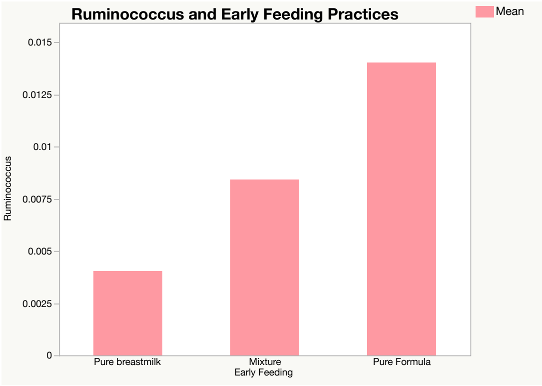

Figure 91: Ruminococcus and antibiotic consumption.

Infants who have been given 4+ doses of antibiotics have higher levels of *Ruminococcus* than infants who have been given fewer doses.

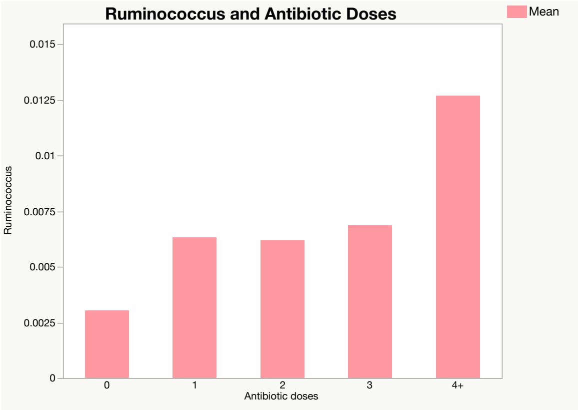

Figure 92: Ruminococcus by early feeding practices.

BF infants have lower levels of *Ruminococcus* than do infants who were FF or MF.

This bacterium was an early colonizer in this population, and was constant through the second year (Figure 90). The study infants however consistently had significantly lower levels than previous studies indicated. Birth mode was not a significant factor in the colonization of these bacteria. Directly contradicting Tannock et al.'s findings (2013), Infants who were breast-fed and significantly fewer *Ruminococcus* than those that were mixed fed (P=.03) and those that were formula fed (P=.0005) (see Figure 92). Infants that received 0 doses of antibiotics had significantly lower levels of *Ruminococcus*, than infants who received 4+ doses (P=.017) (see Figure 91). The trend is robust across the total doses, although not significantly different. These bacteria were significantly and positively correlated with Zwfl in the general population of infants, particularly at 8-11 months and among the AGP infants at 12+ months.

All infants with higher than reference range were either MF or FF, and birth-mode was mixed. Most of these infants also had co-occurrences of other pathogenic microbes. For example, subject 5, at 10 months had 23% Lachnospiraecea, 3.4% of which was represented by bacteria from the genus *Ruminococcus*. At 15 months, this infant acquired high levels of Clostridium (13%). Infant 2, at 9 mo. had 3.6% *Ruminococcus* and previously had high levels of Enterobacteriaceae at 4 months. Infant 45, at 5 months also had high levels of *Ruminococcus*, while simultaneously high levels of *Faecalibacterium*. These findings may indicate dysbiosis in these infant intestinal microbiomes. Taken together, and at least at high levels, this bacterium is likely to have ill effects on infant and microbiome health.

Key words: key successional bacteria, supports growth, prevents malnutrition.

Staphylococcus

Staphylococcus is a genus of Gram-positive bacteria. They are round (cocci) and form grape-like clusters, and include over 40 known species. There is substantial sub-speciation among this genus, containing up to four known subspecies per species (Harris et al. 2002). Most species of this genus are harmless and reside normally on the skin and mucous membranes of humans and other organisms. *Staphylococcus* can cause a wide variety of diseases in humans and animals through either toxin production or penetration. *Staphylococcal* toxins are a common cause of food poisoning, as they can be produced by bacteria growing in

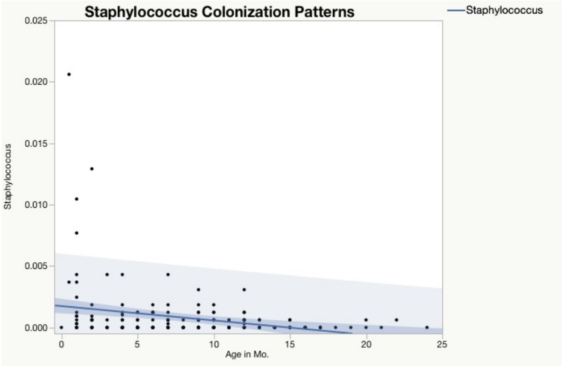

Figure 93: Staphylococcus colonization patterns.

Staphylococcus is an early colonizing bacterium that colonizes the infant intestinal tract (albeit in low levels) during the first days and months of life, but decreases by the 3-4th month.

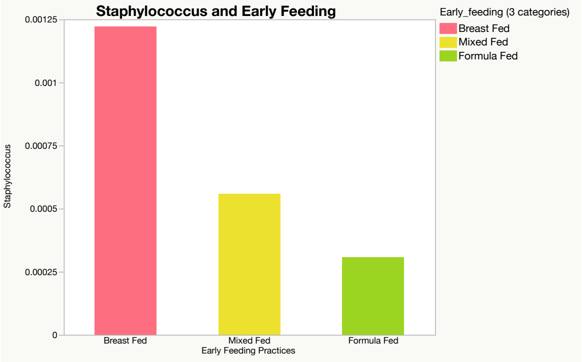

Figure 94: Staphylococcus and early feeding practices.

BF infants have significantly higher relative abundances of *Staphylococcus* than FF infants.

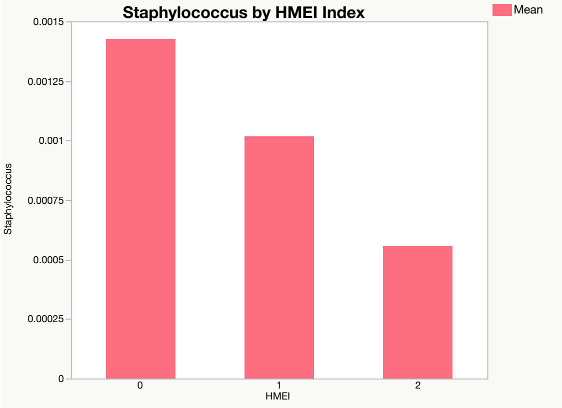

Figure 95: Staphylococcus and HMEI index.

Although not significant, lower HMEI score individuals have higher levels of *Staphylococcus* than infants among the high HMEI group.

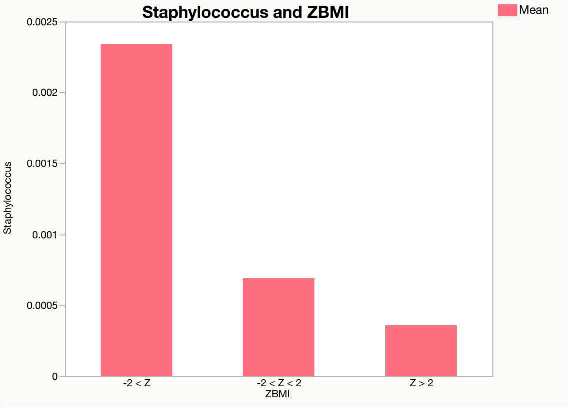

Figure 96: Staphylococcus and ZBMI scores.

UPG infants have significantly higher *Staphylococcus* than infants in the APG or OPG groups (P=.03, F=3.5).

improperly stored food items. Found worldwide, they are a small component of soil microorganisms (Madigan and Martinko 2006).

This bacterium is more common in breast-fed than bottle-fed infants (Lundequist et al. 1985; Balmer and Wharton 1989; Lindberg et al. 2011). Moreover, Vael et al. (2011) found that BMI z-scores between 3 and 26 weeks of age negatively correlated with *Staphylococcus*, controlling for infant feeding and several other risk factors for higher BMI.

Study findings

Staphylococcus was an early colonizer, decreasing in relative abundance with time (see Figure 93). Similar to other infant microbiome studies (Lundequist et al. 1985; Balmer and Wharton 1989; Lindberg et al. 2011), the bacterium was more prevalent in BF and MF infants than FF ones, and were found at the highest relative abundance in the days and weeks after birth. BF infants harbored significantly higher relative abundances of these bacteria than FF infants (P=.05) (see Figure 94). Although this bacterium is an early colonizer, it was not found to be associated with birth mode. Like Vael et al. (2011), I found that these bacteria were negatively correlated with ZBMI scores in this population (P=.03, F=3.5), but not other measures of infant growth (see Figure 96). An overabundance might signal failure to grow and pathogenicity, although in a healthy gut they may be protective from overweight. The consistent finding that this bacterium is found cross-culturally in BF neonates suggests their important role in the early successional gut microbiota, however the functional significance has yet to be elucidated.

Key words: Early colonizer, negative correlation with ZBMI in infants, higher in BF infants, pathobiont.

Streptococci

Members of this genus are spherical, Gram-positive, facultative anaerobic bacteria found in the neonatal intestinal tract. It is one of the first microbes to colonize the digestive tract during a vaginal delivery. *Streptococcus* colonizes the infant gut in the first days after birth, and utilizes the oxygen present creating a reduced environment which allows for growth of obligate anaerobic bacteria like *Bacteroides* and *Bifidobacterium* (Parracho, McCartney, and Gibson 2007). Breast-milk microbiota is also dominated by a few genera including *Streptococcus* (Hunt et al. 2011), offering the infant a steady influx of these bacteria. These bacteria also ferment sugars into lactic acid.

Evidence from one Finnish cohort indicates that the maternal gut microbiome changes during the course of pregnancy. Koren *et al.* (2012) reported that as women progress through pregnancy, bacterial diversity decreases. Overall, the authors found that greater gestational age was associated with the presence of more high-energy-yielding fecal microbiota, which are typically characteristic of microbial communities found in individuals with metabolic syndrome (Koren et al. 2012). Specifically, the proportion of pro-inflammatory Proteobacteria, including species of the Enterobacteriaceae family and *Streptococcus* genus, decreased from the first trimester to the third trimester, while the proportion of anti-inflammatory *Faecalibacterium prausnitzii* increased. These changes were independent of pre-pregnancy body weight, gestational diabetes, diet, and antibiotic use, suggesting that they were due to normal pregnancy-related alterations to the maternal endocrine and immune systems. However, about a third of women have *Streptococcus agalactiae* (also known as Group B *streptococcus* or GBS) in their vaginal microbiomes prior to birth. Although this strain is largely harmless to adults, it can cause serious infections in newborns who acquire it during birth. GBS is known to cause meningitis and sepsis in infants (Bohnsack et al. 2008). For this reason, third-trimester screening for GBS is a standard component of pre-natal care, mothers who test GBS positive are given a prophylactic antibiotic right before delivery, and the babies are born free of infection.

Study findings

Levels of *Streptococcus* start out high in this population and then decrease at a steady rate by the second year of life. Birth mode is not a significant factor, although formula-fed infants have the highest average abundance compared to breast-fed infants (P=.02, F=4.3).

Patterns among infants who had high levels of Streptococcus were difficult to discern. The infants were mostly BF or MF, with one FF. 5/7 of the infants were vaginally delivered. While this may be a key bacterium in the successional development of healthy breastfed infants, higher than predictive ranges of this bacterium were mostly problematic. It is possible that this bacterium is important for healthy niche production and changes in the intestinal environment for the first month or two, after which they become more inflammatory for infant immunity. For example, infant 58, VDMF was under 1 month old at her first sampling. She had high levels of Lactobacillus and Bifidobacterium as well as high levels of Streptococcus. She was quite healthy at this point. The remaining 6 infants with higher than predictive levels of these bacteria were symptomatic of infection, and all were over 4 months of age.

Key words: early successional bacteria (active in niche production), pathobiont, pathogenic

Veillonella

Veillonella are Gram-negative, anaerobic bacteria that commonly inhabit in the intestines, mouth and vagina of mammals (Verma et al. 2010). These bacteria are well known for their lactate fermenting abilities and helps in dehydroxylation of bile acids. Fermentation of lactate to propionate and acetate is by the methylmalonyl-CoA pathway. Species *Veillonella parvula* has been implicated in rare cases of osteomyelitisand endocarditis as well as in causing abscesses, bacteremia, and pneumonia and endocarditis (Marik and Careau 1999; Boo et al. 2005; Brook 2006).

In a recent paper, researchers attempted to use an advanced rule-based algorithm to construct complex microbial regulatory networks that theoretically extract cooperative and competitive relationships between microbes from highthroughput sequencing time series data. This team found that *Veillonella* cooperatively interacted with *Clostridium XI*, *Bifidobacterium* and *Streptococcus*, but competitively with *Bacteroides*. In particular, they found a positive correlation between *Veillonella* and *Bifidobacterium*, both residing in human intestines and oral mucosa as anaerobic commensal organisms (Verma et al. 2010). In addition, *Veillonella* was also observed to have a positive correlation with *Streptococcus*, likely interacting cooperatively during the production and degradation of Lactic acid (Chalmers et al. 2008; Palmer et al. 2006; Kara et al. 2006). In addition, the constructed network suggests that a competitive relationship may exist between *Veillonella* and *Bacteroides*. *Veillonella* and *Bacteroides* are gut-associated obligate anaerobic genera found in maternal feces, breast milk and neonatal feces (Jost et al. 2014); and the difference in their efficiency at different levels of oligosaccharide consumption may suggest they occupy different metabolic niches (Marcobal et al. 2010).

Study findings

Levels of Veillonella decreased steadily over time (see Figure 97). In the current study, breastfed (BF) infants had the highest proportions of this bacterium, although these trends were not significant. Vaginally delivered (VD) infants also tended to have higher levels of these bacteria than did Cesarean delivered (CD) infants (also not significant), and Vaginally delivered, breastfed (VDBF) infants had the highest levels among the subgroups. The subgroup VDBF infants have high levels in their early months (14.25%), and steadily decrease in number over the course of the first year of life to 3.3% at 12 months. CDFF infants in contrast began with low levels from 4-11 months (ranging from 2.15%-3.63%) and yet leapt to 19.99% at 12 months. Two infants in this group pulled the mean (containing 31.4% and 23.7% of *Veillonella* in their microbiomes), however even when taken out, average count was higher than the VDBF sub group, indicating a delayed colonization pattern. *Veillonella* was found to be significantly and positively correlated to growth outcomes in this population in general, but more specifically in the first months of life (0-3 months).

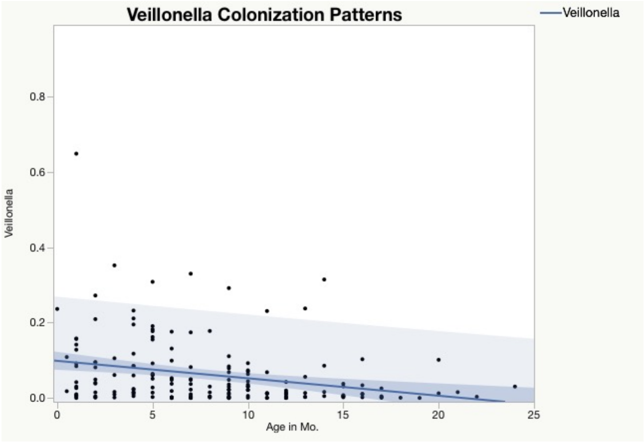

Figure 97: Veillonella colonization patterns.

These bacteria are early colonizers in the infant microbiota, and levels of the bacterium decrease steadily over time.

Among the outlier infants with high abundances, 7/8 were vaginally delivered, and only one infant was FF. The FF infant was subject 5 who had high levels of this bacterium at 14 months (31%), when on average infants at this age had levels under 5%. This late and high levels of *Viellonella* indicate delayed and disrupted microbial colonization. In fact, this infant concurrently had high levels of *Clostridium* (13%), and showed symptoms of diarrhea and GI infection, and would take antibiotics after this sampling.

Key words: lactate fermentation, cooperative relationships with *Bifidobacteria,*

Discussion

This chapter is meant to serve as a guide to the relevant taxa from this Salvadoran study population. It is intended to define current understandings and research regarding each microbe, as well as to illuminate new interactions among microbes, host and environment. This chapter should enhance the other 5 chapters of this dissertation, as well as clarify known and hypothesized functional activities of these taxa as discussed throughout the dissertation.

Through the seemingly chaotic nature of early microbial ontogeny, successional patterns of bacteria emerged. This chapter showed that early care practices such as birth mode, early feeding, antibiotic consumption as well as more proximal eco-cultural variables such as SES and HEMI and number of animals kept in the household, can have a substantial and often lasting influence on what microbes colonize a given infant intestinal community. It also shows that *which* microbes colonize an infant gut, and *when* can have significant effects on infant health and growth. For example, *Faecalibacterium* is a keystone species, considered fundamental to a healthy adult microbiome. This chapter revealed that the early and high level colonization of this bacterium instead produced pathogenic outcomes in infants. *Faecalibacterium* was found to colonize the infant gut earlier in the current study than in previous research, and higher than predictive levels at earlier ages was linked to increased infection rates and increased administration of antibiotics. Additionally, *how* infants acquire the bacterium that became important. In the general population, the middle HMEI index had the highest average levels of this bacterium both at 0-3 mo. and again at 12+ mo., indicating that a moderate exposure is preferable for a healthy age-appropriate colonization of these bacteria. However, many of the infants who had higher than predictive levels of this bacterium had the highest environmental exposures, including exposure to animals, older siblings, and early food introduction. These were the infants who also showed the highest levels of illness, indicating that extremely high levels of this bacterium are linked to ill health. While causal arrows are difficult to determine in an observational study, these findings suggest that observing not only the mean outcomes of a population, but also the outliers helped to describe the pathogenicity or probiotic functions of a given bacterium.

Predictably, I found that many of the early colonizers were associated with early feeding practices, and later colonizers with more environmental factors. For example, Bifidobacterium*, Lactobacilli, Megasphaera, Staphylococcus,* and *Veillonella* were positively associated with breastfeeding, while *Blautia, Clostridiales, Enterobacteriaceae, Enterococcus, Lachnospiraceae,* *Ruminococcus,* and *Streptococcus* were associated with formula feeding. In particular, Enterobacteriaceae and *Blautia* were associated with a powdered cow's milk consumption (which a few mothers used instead of more costly formula). Later colonizers such as *Akkermansia*, *Lactobacillus* and *Prevotella,* were associated with environmental factors including the number of different kinds of animals kept in a household and HMEI scores, whereas *Bacteroides, Lactobacillus, Ruminococcus,* and Lachnospiraceae were found to be sensitive to care practices including antibiotic loads. It should be emphasized that *Prevotella* was associated with environmental outcomes, because current literature links prevalence of *Prevotella* almost exclusively to diet (De Filippo et al. 2010; Wu et al. 2011). This highlights the importance of social and ecological variables in microbiomics research.

Predictive ranges for the main bacteria produced interesting results. Higher ranges of these bacteria had significant growth and health consequences. Higher than predictive ranges of *Akkermansia, Bacteroides, Bifidobacterium*, and *Lactobacillus,* did not produce adverse symptomatology in infants, but rather probiotic affects. Whereas similarly high levels of *Enterobacteriaceae, Enterococcus, Faecalibacterium, Prevotella* and *Ruminococcus,* seemed to produce infectious symptoms and clinical diagnoses. Additionally, I found that certain microbes had close associations to others, accompanied by enhanced probiotic or pathogenic outcomes within the infant microbiome. For example, infants who harbored high levels of *Lactobacillus* also harbored high levels of *Bifidobacterium*, enhancing one-another's probiotic effects in infants of this sub-group. *Blautia* and *Clostridia* also tended to co-colonize infant microbiota with ill-effects. Finally, *Enterobacteriaceae* and Enterococcus seemed to co-colonize the infant gut with amplified pathogenicity.

Conversely, low levels of Lactobacillus in this population may be in part a reason for high levels of illness, while high levels of this bacterium at all ages produced probiotic effects. Future research should explore the causes and consequences of the low levels of this bacterium in this Salvadoran population, particularly given the strong associations of this bacterium to healthy growth phenotypes. If these low levels are found in other similar populations of developing countries, it may be indicative of future health epidemics including allergies, metabolic and autoimmune disorders associated with low levels of *Lactobacillus* during infancy. Future health interventions should not only decrease the prophylactic treatment of antibiotics administered to pregnant women, but also provide mothers and infants with *Lactobacillus* supplementation.

Certain microbes were identified as possible perpetrators of obesogenic phenotypes in this population. For example, formula fed infants tended to have higher levels of *Ruminococcus*, were more likely to have obesogenic phenotypes, and consumed more antibiotics. Conversely, *Staphylococcus* was negatively correlated with ZBMI scores in this population. This bacterium was more common in breast-fed infants than formula-fed ones, and was not associated with adverse health outcomes. Finally, underweight infants have significantly more Staphylococcus than do normal and over-weight infants. It is likely that the colonization of these microbes shape growth phenotypes in this population.

Another pattern that emerged from this chapter was the enhanced effects in the relative abundance of multiple bacteria when infants were both vaginally delivered, and breastfed (VDBF), and/or cesarean-delivered and formula-fed (CDFF). The mechanisms of these multi-factorial, synergistic relationships are unclear, and likely vary among bacteria and infants. For example, even though *Akkermansia* is considered a late colonizer, early care practices seemed to affect whether infants harbored this bacterium and in what quantities. CDFF infants never acquired levels of *Akkermansia* above .33% in their first *two years*. Nearly all infants who acquired higher than predictive levels of *Bacteroides* were VDBF, although neither birth mode nor early feeding were significant independent factors. Additionally, VDBF infants had the highest average levels of *Lactobacillus* (2.62%), whereas CDBF had only .71%, and VDFF only .05%, suggesting that early bacteria seeded through vaginal delivery may affect colonization only when enhanced by breastmilk. Finally, infants of the subgroup VDBF had high levels of *Veillonella* in the early months, tapering off over the course of the first year, whereas CDFF infants experienced a delayed colonization until after the first year of life. Conversely, CDBF infants had the highest levels of *Clostridia* at 0-3 months (11% compared to 2.8% in VDBF infants), although unfortunately CDFF did not have enough of a sample size to analyze. Together these findings suggest that birth mode and early feeding together may help seed and nurture a successional development of the gut microbiome.

Two main limitations to this study deserve consideration. In general, the measure of illness was undependable. At each interview, I asked the mother/caretaker: how many times the child was sick since our last visit, what was the diagnosis (or symptoms), how long did the illness last, and what was prescribed? During analysis, these raw counts of illness episodes was used as proxy for "health" of an infant. Unfortunately, raw counts of illness do not tell the entire story, and in this case, did not translate accurately to the ethnographic data on infant health. This may be for multiple reasons: first, maternal recall of illness is highly variable and undependable. Second, a GI infection in one infant may produce 1 day of diarrhea, and 8 days in another infant, making a raw count of total illnesses an unreliable measure of ill-health. Finally, it is possible that a developing infant immune system requires a certain degree of exposure and illness for a "healthy" regulation. Thus, determining "healthy" children from "unhealthy" proved problematic. For these reasons, analyzing infant outliers who had extremely high levels of certain bacteria proved to be a fruitful exercise, one that invited the weaving of qualitative data to more fully describe the health or illness experience of these infants at the time of high colonization.

Moreover, limited sample size unfortunately did not allow for multivariate analysis among subgroups such as the outlier infants. Multivariate analysis would have allowed me to tease out relationships with more precision, assessing possible confounding variables. As the cost of sequencing decreases and studies can draw from larger populations of infants, these associations should become even clearer. In the meantime, this chapter illustrates the importance of triangulating the early microbial ecology (including care practices), infant gut microbiota and growth/health outcomes, to support the development of locally appropriate health interventions designed to support the successional development of the infant gut microbiome.

Other charts and reference materials:

Quick reference microbial summary:

- Pro-inflammatory: Clostridium, *Enterobacteriaceae,* Streptococcus (depending on age), Prevotella, *Streptococcus*
- Anti-inflammatory: Rothia, (generally), Bifidobacterium, Faecalibacterium (generally), Akkermansia, Enterococcus, Prevotella, Roseburia
- Pathobiont: Blautia, Clostridia, Corynebacterium, *Enterobacteriaceae,* Enterococcus, Lachnospiraceae, Prevotella, Staphylococcus, *Streptococcus, Faecalibacterium(?)*
- Related to growth/obesity/undernutrition: Faecalibacterium, Lachnospiraceae, Bifidobacterium, Lactobacillus, Megasphaera, Akkermansia, Prevotella, Ruminococcus, Staphylococcus
- Milk (HMO) fermentation: Bifidobacterium, Veillonella, Lactobacillus, Bacteroides, *Enterobacteriaceae (cow's milk oligo), Blautia(?),* Megasphaera, Ruminococcus
- Complex carbohydrate fermentation: Faecalibacterium, Bacteroides
- Probiotic effect: Rothia, (generally), Bifidobacterium, Faecalibacterium, Akkermansia, Enterococcus (possible), Lactobacillus, Ruminococcus(?)
- Biomarker of healthy gut: Faecalibacterium (at certain ages and levels), Rothia, Lactobacillus, Roseburia (not in this population)

Patterns of colonization:

First months low prevalence, peak mid to late first year, then drop off by weaning age (12+):

- Akkermansia: delayed then exaggerated colonization in FF
  - Mediates weight gain
- *Bacteroides*: delayed colonization in FF
  - Sugar degradation for nutrition and energy
  - Involved in innate immune response

- *Lactobacillus*: peak in BF is much lower than other western infants. FF infants almost never acquire Lactobacillus.
    - Contribute to digestion
    - stimulate the immune system
    - inhibit the growth of pathogens

First months low but steady growth over time:

- *Faecalibacterium*: delayed colonization in FF infants
    - Anti-inflammatory properties (produces large amounts of butyrate)
    - Major producer of SCFAs
- *Megasphaera*: delayed colonization in FF infants, never getting very high.
    - Produces SCFAs

First months high and steadily trail off over time:

- *Bifidobacterium*: lower levels throughout, evening out in 12+ months
    - Anti-inflammatory
    - Breaks down Oligosaccharides (milk sugars and proteins)
    - Works in concert with lactobacilli to produce immune responses and carbohydrate fermentation
- Enterobacteriaceae: both show similar trends but FF are exaggerated highs and lows
    - Well studied and diverse family of bacteria. Most are types of E. Coli
    - The growth of *E. coli* is suppressed when human milk is present in the digestive tract, because the proteins present in the human milk create a hostile environment for *E. coli*. The majority of *E. coli* produces K- and B-complex vitamins in the intestines, which are absorbed by the infant's body as essential nutrients (20). However, different *E. coli* strains have different functions. Some *E. coli* strains collaborate with other bacteria to breakdown and obtain nutrients.
- Streptococcus: FF infants fluctuate more, but have the same general trend.
    - Pathobiont (can be pathogenic, but can also not be)
    - Ferments sugar into lactic acid
    - Can be inflammatory
- Veillonella: FF infants fluctuate from low to high, then low to high.
    - Milk fermentation
- Rothia: only found in BF and MF kids
    - Anti-inflammatory properties

Low counts throughout first two years:

- Blautia: exaggerated levels in FF
    - may help produce fecal butyrate levels
    - little is known
- Lachnospiraceae: BF fluctuate slightly, FF have exaggerated increases
    - Produces SCFAs
    - Early blooms linked to obesity

Additional figures

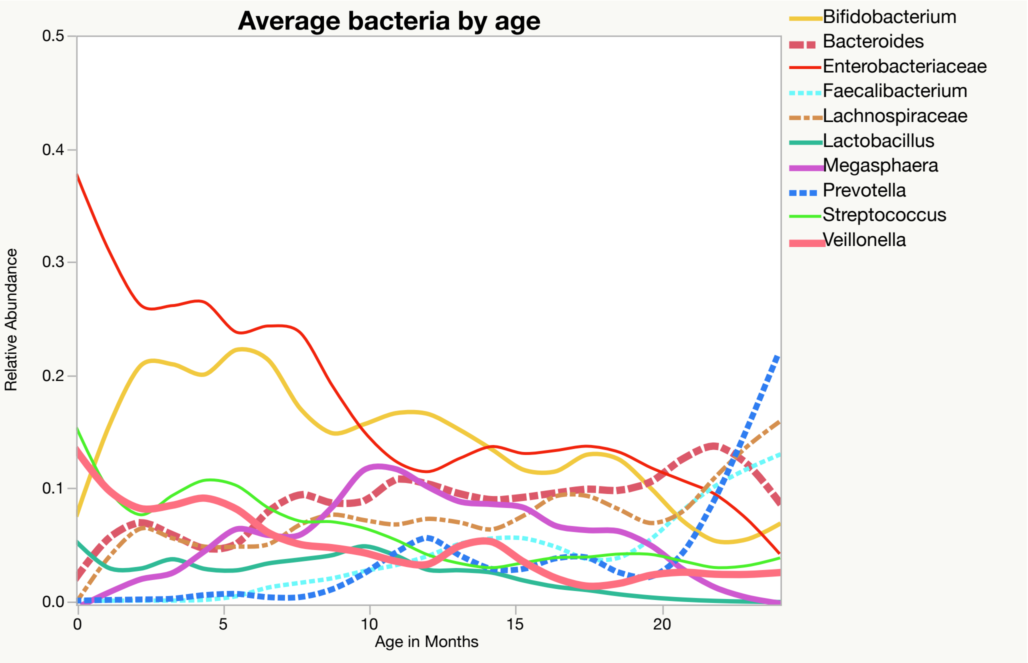

Figure 98: Average bacteria by age

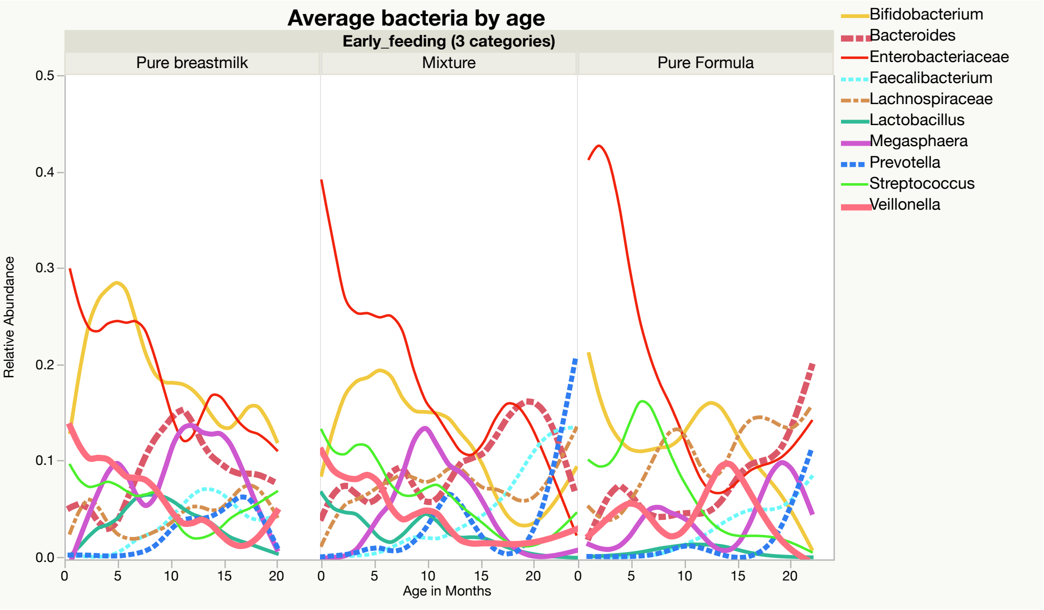

Figure 99: Average bacterial colonization patterns among the early feeding groups.

Relative abundance of top genera in infants of different age groups by early feeding practices.

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CHAPTER 6: CONCLUSION

This dissertation has explored how the early experience and exposure of infancy becomes incorporated into the structure and function of infant biology. The gut microbiome represents a unique anatomical niche: an important interface between an internal, biological, and microbial environment and an external, material world—a space within the human body that requires external input for proper development and maintenance, and in return affects the daily lived experience and relations with the outer world of its human host. For the human gut, this "external input" is an inherited mix of co-evolved microorganisms. This dissertation explored the suite of human behaviors and institutions that alter both vertically and horizontally transmitted sources of microbiota in the Salvadoran village of Joya de Cerén. Findings have shown that even in a relatively homogeneous, resource-poor community in central El Salvador, existing variation among socio-economic classes, households, and care practices had significant effects on infant microbiota, growth and health.

Chapter 1 introduced and provided background for the complex and multi-level investigation to follow. First, a brief background on the development of the infant gut microbiome was provided, and hypotheses were outlined regarding the vertical and horizontal exposures of early childhood. Next, a brief cultural and political history of El Salvador and Joya de Cerén provided an understanding of both the unique nature of this field site but also what makes it a generalizable model for the exploration of microbial ecologies within other developing populations. A description of the population was followed by an elaboration of the longitudinal study design and novel measures of microbial ecology and ethnographic data collection. That chapter established the view that to understand infant biology and health requires exploration of the social, political and ecological context in which it developed.

Chapter 2 explored the basis of vertical microbial selection by focusing on maternal-infant interactions. Comparisons between vaginally and cesarean birthed infants showed how medical practices interrupt the initial seeding of maternal vaginal flora, although effects were not as pronounced in this population as in others. Nevertheless, vaginally delivered infants harbored higher abundances of *Akkermansia, Bifidobacterium*, *Lactobacillus*, and *Megasphaera* compared to CD infants, illustrating that birth mode may affect infant microbial development and function, even into the second year of life when overall community composition and alpha diversity appear similar among birth modes.

As predicted, early feeding practices were associated with pronounced differences in microbial ontogeny, and the cessation of breast-feeding seemed to explain more of the variation in community composition than did the introduction of solid foods. This was particularly true in breastfed infants who exhibited relatively little disruption in their community composition when solid foods were introduced, compared to formula feeders who experienced pronounced changes with the introduction of complementary foods, in agreement with other recent studies (Bäckhed et al. 2015; Thompson et al. 2015).

This chapter also found that birth mode and early feeding operated as synergistic factors associated with enhanced vertical selection of microbiota. Vaginally delivered, breastfed infants had higher levels of key anti-inflammatory bacteria such as *Akkermansia*, *Bifidobacterium, Faecalibacterium, Lactobacillus* and *Bacteroides*, while Cesarean delivered, formula fed infants experienced delayed acquisition, or some cases never acquired these key microbes. The latter group acquired significantly higher levels of pro-inflammatory bacteria such as *Streptococcus*, *Blautia*, and Lachnospiraceae. *Lactobacillus* was discovered to be nearly absent in this population in general, although the highest levels of relative abundance was found in the VDBF group.

Chapter 3 broadened the scope of investigation to horizontal patterns of selection, including social, political economic and ecological variables that influence infant care and microbiota development. This chapter evaluated the hypothesis that differences in household ecologies (shaped by broader socio-political and economic forces) would produce differential exposures and therefore variation in microbiome composition and development. Findings illustrated that large-scale structural changes in socio-political and economic conditions in the country have affected labor markets, demographics, social networks, gender relations, and household socioeconomic conditions in this Salvadoran village. The burden of both formal sector jobs and child-rearing falls squarely on young women of child-bearing years. Factory jobs are inflexible, with long hours and low pay. Consequently, gendered labor practices resulted in significant differences in infant microbial ontogeny. Infants with working mothers harbored significantly higher α-diversity and less microbial stability, with increasingly inflammatory bacterial profiles. Specifically, these infants harbored higher levels of *Blautia* and Lachnospiraceae, and lower levels of key probiotic bacteria such as *Bifidobacterium*. Working mothers reported that their infants had fewer total illnesses and slightly less antibiotic treatment compared to unemployed mothers. By contrast, with higher levels of inflammatory bacteria and relatively volatile colonization patterns, infants of working mothers very likely experienced more illnesses. These findings may be due in part to the excruciatingly long work days and weeks of factory workers. Mothers work 12 hour days, 6 days a week, with no family leave days available. These mothers simply do not have the time to care for their sick infants, thus they likely under-report illnesses compared to stay-at-home mothers. It is also possible that the waking hours with infants are so short that working mothers are less aware of the illnesses their infants experience. Additionally, clinic visits are often day-long events, making it less likely that a caretaker will take an infant to the clinic, resulting in lower reported and treated illnesses.

Another important finding in this chapter was that the Household Microbial Ecology Index (HMEI) was an important indicator of early microbial exposures and likely ontogeny. In the early months of life, low HMEI infants (highest exposures) were found to have the highest levels of α-diversity compared to the middle or high HMEI groups. In the later months when α-diversity would be presumably beneficial, the middle HMEI group were found to harbor the highest levels of α-diversity as well as many anti-inflammatory, protective bacteria including *Lactobacillus*, *Faecalibacterium*, and *Roseburia*, and bacteria that are known fermenters of breast-milk including *Bacteroides, Lactobacillus, Megasphaera,* and *Veillonella*. This middle group also harbored the lowest levels of inflammatory microbes including Lachnospiraceae and *Streptococcus*. Overall these findings suggested that too much or two little horizontal exposure can negatively affect infant microbial development. This is a surprising result in a semi-rural village with relatively homogeneous socio economic resources.

Chapter 4 investigated the hypothesis that the developing microbiome evolved in part to support high infant energetic demands in early months of life. Additionally, it proposed that a disrupted colonization pattern during early infancy would be reflected in divergent patterns of infant growth. If true, comparative analyses among different growth phenotypes (defined by weight-for-length Z-scores), can help describe the parameters of a "healthy" population-specific infant microbiome. Analyses revealed a growth-related range of alpha diversity in the first few months of life, where low diversity was associated with diminished growth outcomes, and high diversity was associated with overweight outcomes (see Table 31). This association shifted at 12+ months, where AGP infants had the highest α-diversity compared to the other two groups. These results lent further evidence to the hypothesis that in contrast to the adult microbiome, α-diversity in the first few months may not be desirable (Sheets and Worthman 2014, Thompson et al. 2015).

As hypothesized, formula-fed infants suffered the highest rates of illness, were prescribed the most antibiotics and yet had significantly higher growth scores than mixed-fed and breast-fed infants. Under scrutiny of a diligent public health promoter who worked in this population, levels of subclinical infection likely did not go unnoticed for long enough to influence growth trajectories (other than among small subgroups). Aside from established literature showing associations of macronutrient profiles in formula with overweight growth phenotypes (Dewey 1998; Dewey 2003), this chapter pursued the hypothesis that higher antibiotic loads may also encourage community assembly toward a more metabolically active profile. In all, this chapter described the successional and metabolically active nature of the infant microbiome. Different assortments of microbes were associated with growth at different ages, suggesting their roles in shaping growth outcomes. Growth phenotypes in turn, support the understanding of "healthy" microbial profiles.

Contributing a key element to relationships between maternal labor patterns and infant development, Chapter 4 clarified how infants of working mothers were found to be significantly smaller than infants of mothers who were unemployed. The effects were most pronounced in the lowest SES level. These findings can be in part explained by discoveries from Chapter 3: mothers worked insufferable hours, were poorly paid, and were prohibited from exclusive breastfeeding (and in all but one case, were prohibited from breast pumping). Additionally, lower SES households rarely had refrigeration, making breast-pumping problematic or impossible. A month's salary at a local factory was equivalent to a month's supply of infant formula (the costs of daycare not included). Thus, the poorest mothers supplemented their infants with cheap liquids or heavily watered-down formula in the long hours of their absence. Alternative bottle-feeding selected for discrete microbial signatures, and highly unstable profiles with pro-inflammatory gut bacteria. Missing macronutrients, unhealthy successional gut communities, and possibly subclinical infections likely contributed to adverse growth and health outcomes among these infants. In all, this chapter contributed both statistical and qualitative evidence about how broader social, political and ecological processes alter the microbial and somatic development of infants in this Salvadoran population.

Development of the infant microbiota entails an immensely complex succession of organisms that differ in function, individually and as an assembly. As an aid to parsing this complexity, Chapter 5 provided a guide to the most common and functionally important microbes in this population. The chapter described current understandings of the form and function of each of the most relevant bacteria. Study data for each microbe were then analyzed to identify colonization patterns, vertical and horizontal exposure associations, and associations with growth or health outcomes. Predictive ranges based on age-specific distributions in the study population proved to be particularly helpful in identifying outliers in microbial colonization patterns of most bacteria. Ethnographic data were utilized to discover individual stories behind outliers, helping to expose complex and sometimes situationally-dependent pathogenic or probiotic interactions. This chapter substantiated findings from other chapters, as well as explored novel connections within the three-fold host-microbe-environment microbial ontology.

Surprising discoveries were made about microbe-ecology interactions. Unlike current literature linking *Prevotella* to diet (De Filippo et al. 2010; Wu et al. 2011), the occurrence of these bacteria were primarily associated with household and environmental exposures. After controlling for age, infants from low-HMEI households (with the highest environmental exposures) had significantly higher levels of *Prevotella* than infants in the other two groups. SES level was not a predictor of colonization by this bacterium, suggesting that material environmental exposure was likely responsible for higher levels of this bacterium. Although not significant factors, increased levels of these bacteria were found in infants who lived in houses with higher population density, and among more types of animals. To the best of my knowledge, no study has previously explored links between early developmental ecologies and *Prevotella* colonization among infants. Future research should explore these findings in adults, to discover the effect of ecological factors on the mature microbiome. *Faecalibacterium* was positively associated with a middle-range HMEI score, but outlier levels of this bacterium (high levels too early and low levels in the second year) were associated with the highest environmental exposures, increased infection rates, and higher loads of antibiotics. This shows that there likely are age-dependent ranges for not only alpha diversity, but also for pathogenicity of certain microbes. This latter finding highlights the complexity of microbe-host-ecology interactions in illness and health outcomes. *Bacteroides* were found to be particularly sensitive to antibiotic administration. *Faecalibacterium* was positively associated with a middle-range HMEI score, but outlier levels of this bacterium (high levels too early and low levels in the second year) were associated with the highest environmental exposures, increased infection rates, and higher loads of antibiotics. This shows that there are likely age-dependent ranges for not only alpha diversity, but also for pathogenicity of certain microbes. This latter finding highlights the complexity of microbe-host-ecology interactions in illness and health outcomes. *Bacteroides* were found to be particularly

sensitive to antibiotic administration. Levels of this bacterium did not recover after the first dose of antibiotics in this population.

Other bacteria showed probiotic effects in outlier infants, suggesting that higher than average ranges were beneficial for infants. These bacteria included *Akkermansia, Bacteroides, Bifidobacterium*, and *Lactobacillus*. Conversely, high levels of *Enterobacteriaceae, Enterococcus, Faecalibacterium, Prevotella* and *Ruminococcus,* were associated with infectious symptoms and clinical diagnoses. Certain microbial interactions were associated with enhanced probiotic or pathogenic outcomes within the infant microbiome. For example, infants who harbored high levels of *Lactobacillus* also harbored high levels of *Bifidobacterium,* reciprocally enhancing their probiotic effects in infants of this sub-group (positive health outcomes were demonstrated through ethnographic observations and caretaker interviews). *Blautia* and *Clostridia*, as well as *Enterobacteriaceae* and *Enterococcus* were associated with amplified pathogenicity. Together these analyses traced individual microbes, outlier infants and identified distinctive patterns of exposure that were related to infant health and growth outcomes.

Other patterns emerged that span multiple chapters, largely related to the original hypotheses outlined in the introductory chapter:

> **H1*:*** The optimal ontogeny of the gut microbiome requires first a vertically-selected microbiota. As horizontal exposures increase, and with the continued protection of breastmilk over the first year(s) of life, the gut microbiome diversifies and stabilizes.

> **H2:** Contemporary human behaviors can interrupt the timeline of both vertical and horizontal exposures, resulting in altered microbial assembly, growth and health outcomes.

The (**H1**) hypothesis that a highly-selective, vertically-transmitted microbiota would be initially optimal for infant immunity, growth and development, was first evidenced by exploring patterns of α-diversity among the study population. Infants that were initially seeded through vaginal delivery experienced a more stable gradient colonization pattern of α-diversity, than cesarean delivered infants. Breastfed infants had lower initial α-diversity than formula fed infants in the same age range, supporting the idea that breast milk helps select key microbiota through both prebiotic (Donovan et al. 2012; Kunz et al. 2000; Asakuma et al. 2011) and antimicrobial factors (Praveen et al. 2015; Walker and Iyengar 2015). Breastfeeding also supported the stability of α-diversity over time. BF infants showed a more constant increase in diversity, even after the introduction of complementary foods, compared to wild fluctuations in FF infants. This early evidence supported the hypothesis that vaginal delivery and breastfeeding were evolutionarily designed behaviors to support the initial and continued transfer of a specific, maternal microbiota to offspring. The umbrella protection of a maternal breastmilk microbiota seemed to have supported the development and stability of the developing microbiome even during the intrusion of otherwise disruptive horizontal exposures. Thus, contemporary cultural practices such as formula feeding and cesarean delivery altered and destabilized the vertical inheritance of microbiota in this population of infants (providing evidence for **H2**).

But what taxa makeup this early selection of microbiota? In this population, as in the literature, the maturation of microbiota was a nonrandom process where distinct signature species were identified at different ages. The first microbiota to colonize the infant gut were from the Firmicutes phylum (anaerobic *Streptococcus*, *Staphylococcus* and facultatively aerobic *Veillonella*), and Proteobacteria (facultatively anaerobic bacteria including Enterobacteriaceae). These bacteria have been shown to structurally alter the infant intestinal tract, making the luminal environment hospitable for the subsequent colonization of anaerobic bacteria (mostly from the Actinobacteria phylum including *Bifidobacteria*) (Adlerberth and Wold 2009). The next succession step is the increase in the Bacteroidetes phylum of bacteria, as levels of Actinobacteria and Proteobacteria decrease. These findings support the hypothesis of an early and highly selected for, successional transfer of microbiota in the current study population.

As with patterns of α-diversity, community composition also differed significantly by contemporary early care practices including cesarean sections and formula feeding, providing further support for **H2**. In the first 3 months of life, vaginally delivered infants harbored higher levels of *Lactobacillus* and *Streptococcus,* and lower levels of *Clostridium* than cesarean delivered infants. During these months, breastfed infants harbored substantially higher levels of *Bifidobacterium*, *Lactobacillus*, *Veillonella,* and *Staphylococcus,* and only half the levels of Enterobacteriaceae compared to FF infants. FF infants harbored undetectable levels of *Lactobacillus* in this age range. These results showed that CD and FF infants contained lower levels of these key early bacteria, and/or that they had delayed colonization, either of which can functionally effect infant gut development. Moreover, FF infants of both birth modes experienced volatile diversity patterns across successive sampling points, providing more evidence that breastfeeding contributes to a more stable development over the course of the first year.

Interestingly, in combination, the two early vertical inheritance factors tended to show significant associations with microbial signatures even as infants grew older. For example, CDFF infants were found to have significantly higher levels of pro-inflammatory bacteria such as *Streptococcus*, Lachnospiraceae and *Blautia*. While *Streptococcus* was an early colonizer in VDBF infants with no adverse symptomatology, it was associated with high infection rates in CDFF infants in the middle of their first year, illustrating that delayed colonization patterns can have deleterious effects on infant development and health. Conversely, older VDBF infants harbored higher levels of anti-inflammatory bacteria such as *Akkermansia*, *Bifidobacterium, Faecalibacterium, Lactobacillus* and *Bacter*oides all of which are expected in infants of these age groups, and yet were delayed in CDFF infants.

H1 and **H2** were further supported by findings of an individual genus that were seeded through vertical inheritance yet influenced by horizontal exposures. The genus *Akkermansia* is a biomarker for a healthy adult gut. These bacteria stimulate the regeneration of the gut mucosal layer and thus initiate a positive feedback loop between host and microbe (Belzer and de Vos 2012), and have been shown to initiate colonization within the first month, yet only doubling in relative abundance around 6 to 12 months (Collado et al. 2007; Midtvedt et al. 1994). In the current study population, vertical transmission factors were associated with later colonization patterns, suggesting that these bacteria were either seeded early, or benefited from the successional processes of early colonizers. While the sample sizes were too small to test significance in the early months, CD infants showed a delayed colonization pattern of *Akkermansia* and did not catch up to VD infants until the end of the first year (8-11 months), a trend reflected also in the feeding groups. Most striking, CDFF infants never acquire levels above .33% throughout their first two years. These findings lend support to **H1**, in that *Akkermansia* was seeded early and supported by vertical inheritance, increasing population size as the mucosal lining matures. Importantly, *Akkermansia* were sensitive to horizontal and ecological exposures: infants of households with more types of animals had lower average relative abundance of these bacteria than did infants of households with fewer types of animals. Additionally, there was a trend towards higher levels of *Akkermansia* when infants had lower levels of α-diversity, after controlling for age, although the difference was not statistically significant. Although *Akkermansia* has been shown to be protective against obesity in adults (Everard et al. 2013), the bacterium was positively correlated with growth (weight for length) at age one year in this study population, suggesting an early functional significance. Finally, despite *Akkermansia* being considered a late colonizer, early care practices (cesarean section and formula feeding) affected later colonization patterns, influencing growth and health outcomes of these infant sub-groups. While it is unclear if these associations are because of early seeding, or because early successional processes created a more hospitable environment for this genus, or both, it exemplifies a genus that is influenced by both vertical and horizontal exposures.

Finally, the genus *Faecalibacterium*, is an example of bacteria that were found to be horizontally selected. Known for its anti-inflammatory properties, *Faecalibacterium prausnitzii* is considered a keystone species in the adult gut. In the present study, this bacterium was not associated with early selection factors, and it only reached functionally relevant levels by the 7th month. As in other infant populations (Blanton et al. 2016; Koga et al. 2016), the current study population attained their highest relative abundance of the bacterium by 21 months. Curiously, infants in the middle HMEI group had significantly higher levels of the bacterium than infants of the high HMEI group, suggesting that horizontal exposure is important for the acquisition of this bacterium. The low HMEI group also trended towards lower levels of relative abundance compared to middle HMEI infants (although not significant), suggesting that like *Akkermansia*, a middle range exposure may positively support colonization of this probiotic, anti-inflammatory bacterium. Importantly, the relative abundance of these bacteria was also significantly associated with improved growth outcomes at 0-3 months and again at 12+ months despite their later colonization patterns.

Other broader, socio-political and ecological factors revealed clues about the interruption of both vertical and horizontal exposures. As previously discussed in Chapter 3 and earlier in the conclusion, changing labor markets now demand a young and female workforce. Rigid schedules and long hours prohibited the possibility of exclusive breastfeeding, yet low pay constrained the affordability of infant formula, thus deleteriously affecting the early feeding practices of infants. Moreover, infants of working mothers were often exposed to multiple material environments when they were left with different caregivers. Working mothers were also less available to take a sick infant to a doctor due to demanding work schedules, likely resulting in higher and longer morbidity rates in these infants. These interactions directly and concretely affected both vertical and horizontal microbial colonization patterns: infants with working mothers harbored significantly higher α-diversity, less microbial stability, and increasingly inflammatory bacterial profiles. Specifically, these infants harbored higher levels of *Blautia* and Lachnospiraceae, and lower levels of key probiotic bacteria, including *Bifidobacterium*. Moreover, these variations in microbial colonization were associated with different growth patterns. Infants of working mothers were found to be significantly smaller than infants of mothers who were unemployed. The effects were most pronounced in the lowest SES level. Thus, subclinical disease burden, high levels of ecological exposures, alternative early feeding practices, likely contributed to adverse growth and health outcomes among these infants. As previously discussed, multiple microbes that represent biomarkers of a healthy gut, including *Akkermansia* and *Faecalibacterium*, required a middle-level environmental exposure. Infants of working mothers were more likely to come from either high or low HMEI households, both of which had less-stable microbial profiles, and decreased growth outcomes.

Ultimately, these findings suggest that this multinational labor tendency of hiring young women had a deleterious effect on infant microbial colonization, affecting both vertical (breastfeeding) and horizontal (increasing exposures, influencing care practices).

The Household Microbial Ecology Index (HMEI) was an important indicator of early microbial exposures, microbial ontogeny, health and growth outcomes. As expected, infants in their first three months who were exposed to the messiest environments had higher levels of α-diversity compared to those in the middle or low exposure groups. Conversely, towards the end of the first year, when α-diversity would presumably be beneficial, the middle HMEI group was found to harbor the highest levels of α-diversity as well as many anti-inflammatory, protective bacteria including *Lactobacillus*, *Faecalibacterium*, and *Roseburia*, and bacteria that are known fermenters of breast-milk including *Bacteroides, Lactobacillus, Megasphaera,* and *Veillonella*. This middle group also harbored the lowest levels of inflammatory microbes including Lachnospiraceae and *Streptococcus*. Moreover, the HMEI scale was also found to be a significant predictor of growth outcomes. Children from households that have middle ranges of HMEI experienced better growth outcomes compared to low and high HMEI groups. These associations held across all age groups, suggesting that a middle-level of exposure may be optimal for infant microbial development and growth. Taken together, this evidence suggests that optimal microbial development begins as a highly selective process that diversifies over time, meanwhile protected by breast milk over the course of the first year(s) of life. Together these analyses contribute to understandings of vertical and horizontal exposures as well as the human behaviors, socio-political conditions and events that obstruct them. These findings connected individual bacterial analyses to proximal explorations of household microbial ecologies to macro conditions of gendered labor markets.

In summary, through an innovative triangulation of methodologies, disciplines and theoretical frameworks, this dissertation nuanced current theories, contributed novel discoveries, and initiated a framework for the application of population-specific microbiomic-based interventions. The following paragraphs detail the principal findings of this dissertation and how they contribute to the disciplines and the theoretical frameworks utilized in these chapters.

Until now, developmental microbiomics research has focused on only a few main factors that affect infant microbial development, specifically birth mode, early feeding and antibiotic administration. For this Salvadoran population, and possibly many others, these factors did not account for as much of the variation as other ecological, social and political variables. Instead, previously unexamined factors in the microbiomics field, yet common variables for human biologists, were significantly more powerful in explaining variations in microbial diversity than these previously studied mediators. These key explanatory variables included the ecologies of early childrearing, maternal labor patterns, and demographic transitions, and together highlighted a currently unexplored, yet critically important direction for future developmental microbiomics research.

An integrative model for infant microbial colonization was constructed illustrating these key discoveries and the relationships among them (see Figure 100). The model defines a concrete pathway of embodiment that links global to local to micro to biological processes. Specifically, this model traces the effects of exploitative labor practices of multi-national corporations in El Salvador on community and family gender dynamics, and childcare practices, mediating microbial exposures, thereby shaping infant gut microbiome development and ultimately growth and health outcomes (see Figure 100). The importance of everyday experience and exposure at the household level is closely examined in this model, as they affect microbial development. Moreover, these connections

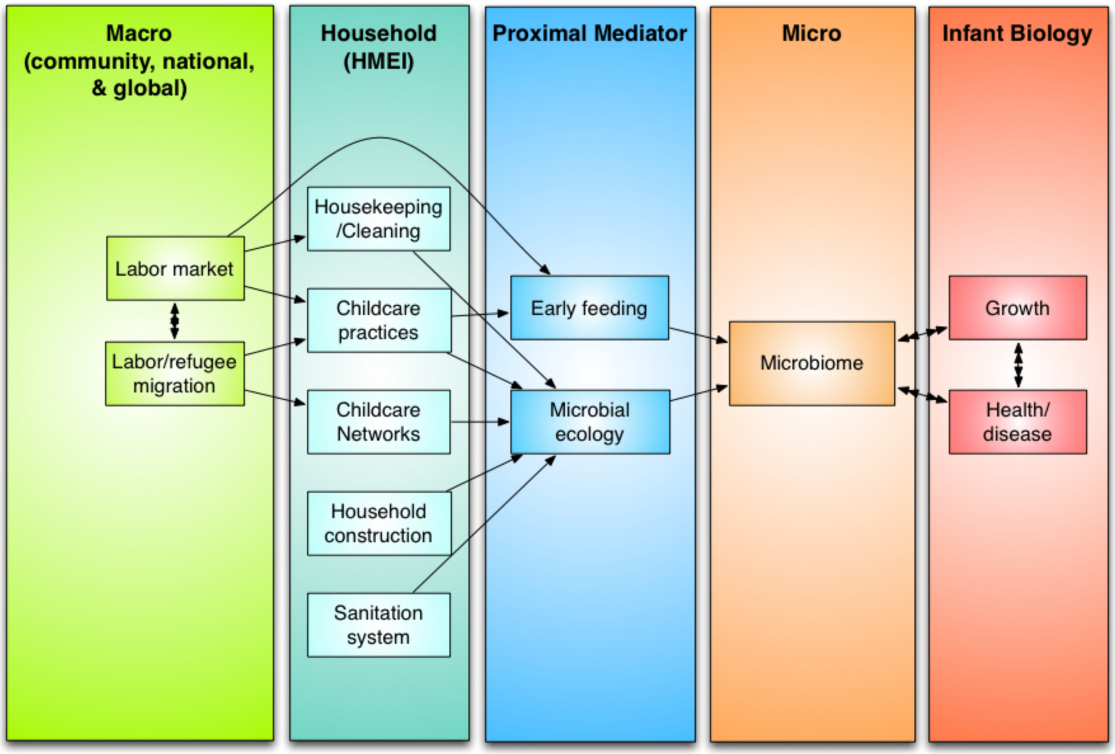

Figure 100: Model of the main findings. This model draws tangible links between the globalization of markets, household dynamics, infant microbial development and growth/health outcomes.

between global/macro processes and the micro-biologies of infants underline a microbe-mediated inheritance of poverty and illness, where each generation inherits a cumulative loss of biodiversity, related biological functions, and even social capital (Adair et al. 2013; Martorell et al. 2010). This complete model is the basis for understanding these deleterious, yet otherwise "invisible" connections that are experienced globally by many mothers, infants and families. Future research can elaborate upon this model.

This dissertation also contributed nuanced understandings to current public health and epidemiologic research. The hygiene hypothesis claims that in high-income, developed nations, improvements in sanitation, hygiene, and control of infectious diseases has had an unfortunate side effect of decreasing exposure to infectious pathogens, parasites, and symbiotic microorganisms that may support the early regulation of immunity (Strachan 1989; Strachan 2000; Sheikh and Strachan 2004). This lack of exposure to commensal and even pathogenic microorganisms has purportedly triggered the increased allergenic and chronic inflammatory conditions occurring in adulthood (ibid). Exposure to these ubiquitous agents is postulated to help in the development of the T regulatory response that when absent in industrialized nations results later in the manifestation of allergies and an array of autoimmune diseases such as inflammatory bowel disease, multiple sclerosis, and type 1 diabetes (Yazdanbakhsh et al. 2002; Yazdanbakhsh and Matricardi 2004).

Findings in this dissertation contribute more intricate explanations of the hygiene hypothesis, showing that the timing and level of exposures matter, particularly in developing countries. At least in this study population, a lower range of alpha diversity is key during the first postnatal months, promoted by a middle-range exposure environment was ideal for infant microbial development, growth, and health outcomes. Infants with too little exposure experience diminished growth and health outcomes as the hygiene hypothesis describes, however infants with high levels of exposure also experience adverse effects in this early developmental period. It is the middle-range infants who receive a foundational, vertically associated microbiota that by the end of the first year of life results in the most diverse microbial communities, and ideal growth and health outcomes. This work underlines the importance of context, timing and degree of exposure during development.

This work also reexamines common microbiomics assumptions. Principally, by showing that there was an “ideal” range of microbial exposures and α-diversity associated with “normal” growth outcomes during specific developmental windows, this finding contradicts the general assumption that high α-diversity is preferable, and encourages a closer examination of the timeline of microbial colonization. Second, that these “normal” growth outcomes were associated with the highest levels of α-diversity by the 12th month and beyond, lends credence to the assumption that high diversity is ideal in a mature, but not developing microbiome. This mid-range of HMEI exposure was associated with higher levels of anti-inflammatory, and vertically-transmitted microbiota both in the early months, and even as diversity was highest among this group by month 12+. These findings point to the importance of investigating timelines of development.

In terms of application, this work developed multi-method analytic techniques to zero in on what a healthy microbiome looks like during different stages of development. Utilizing multiple key factors (birth mode, feeding method, microbial profiles, health histories, growth phenotypes, and ethnographic data), I triangulated data both to validate individual results, but also to capture different dimensions of these complex relationships (Health and Medicine 2014). The result of these analyses was the elaboration of tools that support the comprehensive understanding of ideal microbial development over time, and the related infant care practices. These analytic methods can be utilized to discover biomarkers and ranges for other populations to support effective intra-population interventions. To my knowledge, no previous work has attempted such analytics with these data points, to date.

Finally, with its inherently interdisciplinary framework, there was novel cross-talk among disciplines and contributions to sub-disciplines that invites consideration. First, this dissertation demonstrated the contribution of anthropological and human biological methods and theoretical frameworks to the field of developmental microbiomics. Principally, this study is the first of its kind to longitudinally document the early developmental ecologies of infant microbial development with both longitudinal biological and robust ethnographic data. The anthropological perspective can contribute a wider array of social, political economic and ecological variables to the microbiomics effort. As this research shows, anthroplogy's deep ethnographic, and biocultural methodology can contribute substantially to this nacent field of investigation. Until now much microbiomics research has investigated only cross-cultural comparisons among populations, treating populations as units rather than investigating the substantial variation within.

Understandings of the microbiome also contribute a novel biomarker to biological and biocultural anthropology, subfields that have long been interested in relationships among culture, health and biology. The microbiome seemingly represents a mediator of how social processes and artifacts become embodied, and in return how this embodiment impacts cultural practices and daily lived experience. Understandings of developmental microbiomics can also contribute to research that shows how broader global processes can affect individuals in their daily lives. Findings related to the effects of a transnational labor market on household composition, demographics, and childcare contribute to the anthropology of globalization and labor. How these global and structural dynamics operate on household-mediated childcare practices and early feeding, and thus microbiota and infant growth, is a contribution to biocultural medical anthropology.

Within developmental microbiomics, this dissertation also contributed to a growing body of research looking at how early metabolics may be connected to early development. Moreover, this biocultural investigation chartered new territory in hand with a small but growing body of research that examines the microbial correlates of metabolic conditioning during the early months of life. Findings in this study supported the hypothesis that human-associated gut microbiota are important for human growth and development, and that they may represent a key pathway linking early environments with social and ecological variables to later metabolic and health outcomes (Edwards and Parrett, 2002). Together, these findings represented interdisciplinary cross-talk that supported both the ambitions of the dissertation, but also hopefully a model by which the fields of microbiomics and human biology can meet in the future discoveries of this exciting and dynamic field of research.

Limitations

A major limitation of this study was sample size. A small village population meant that in order to have a large enough cohort, I had to enroll infants of different ages at baseline (ranging from 0-8 mo., averaging 4 mo.) This baseline age-range complicated longitudinal analyses, whereas a typical cohort study would have more precise longitudinal analyses of changes based on specific ages. Instead I used age ranges that were less precise.

Limited sample size also made certain statistical analyses (particularly multivariate analysis) impossible when dividing infants into subgroups. Multivariate analysis would have allowed me to tease apart relationships with more precision, assessing possible confounding variables. The cost of microbial sequencing, although considerably less expensive than previous decades, remains a constraint. As the cost of sequencing decreases and studies draw from larger populations of infants, these associations should become even clearer. At the same time, this study followed nearly 100% of the infants under one year of age in this community, which allowed for a comprehensive exploration of the range of issues for this community of infants at all sampled ages from a cross-sectional and in some instances, longitudinal perspective.

The HMEI scale was developed post-hoc during analysis, as a measure of microbial exposure. While certain of the multiple variables comprising the scale were measured through interview and caretaker reporting, other aspects that were not culturally appropriate to ask about, were drawn from observation. Thus, the overall scores were negotiated among these different variables, with the help of the local health promotor and our research team. Surprisingly, there were no disputes about where the different participants fell on the three-point scale, yet it seemed that there was no single factor that influenced overall exposure; rather, a mix of different factors that determined where a household was placed on the scale. Future research may be able to elaborate, disaggregate and validate the selected (locally appropriate) variables, which was not possible in this context. For these reasons, however, results of this scale should be interpreted with some caution, as this may be an ecological fallacy.

Another limitation was the use of fecal samples to represent the gut microbiome, which may not accurately represent the actual colonization patterns of the large intestine (Zoetendal et al. 2006). As most other studies that focus on gut microbiome, the current study utilized infant fecal matter to represent the microbial composition of the large intestine. Unfortunately, as with any DNA-based, culture-independent study that does not discriminate between live and dead bacteria, the number and identity of bacteria detected in this studies may not represent actual bacterial counts, and should be interpreted with some caution (Zoetendal et al. 2006).

Moreover, the selected method of measuring disease burden was undependable. At each interview a health history was recorded, where caretakers were to recall infant illnesses since the prior visit. During analysis, raw counts of illness episodes were used as proxy for infant health. Unfortunately, raw counts do not tell the entire story, and in this case, did not map accurately into the ethnographic data recorded on infant health. This may be for multiple reasons: first, maternal recall of illness is highly variable and undependable (Burakevych et al. 2015). Second, a GI infection in one infant may produce 1 day of diarrhea, and 8 days in another infant, making a raw count of total illness an unreliable measure of symptomology, severity and possible effect on infant somatic resources or microbial development. Finally, it is possible that a developing infant immune system requires a certain degree of exposure to even pathogenic microbes for the proper regulation of immunity (McDade 2005; Rook 2009; Rook et al. 2013). Thus, determining “healthy” children from “unhealthy” proved problematic. For these reasons, analyzing the ethnographic accounts of infants who had outlier levels of specific bacteria proved more fruitful, one that invited the weaving of qualitative and quantitative data to more fully describe the health or illness experience of these infants during microbial development. Such findings also emphasize the importance of triangulating multiple biomarkers of disease (microbial, growth and health markers) to understand complex relationships between health, growth and microbial development.

With these limitations in mind, this dissertation illustrates the importance of triangulating among early microbial ecology (including care practices), infant gut microbiota, and growth/health outcomes, to develop understandings of intra-population healthy and unhealthy microbial development. These triangulation methods helped to tease apart relationships but also to validate or invalidate statistical associations.

Implications and recommendations for policy

Findings from the study suggest various policy recommendations. The following recommendations are tailored for the Salvadoran government, Salvadoran Ministry of Health, Dr. Julio Cesar Ruiz the vice-director of the medical school at La Universidad de Dr. Jose Matias Delgado, Dr. Yeerles Ramírez, director of Hospital Nacional San Rafael, as well as Salvadoran-based foreign investment companies. Although the following recommendations are for these individuals and institutions, many of the proposals are generalizable to governments and biomedical systems within other developed and developing countries. Moreover, these policy recommendations (particularly true of the proposals regarding birth mode and early feeding) build upon a long history of global public health efforts. Understandings of the development of the infant microbiome will hopefully contribute additional motivations and garner additional support for these efforts. At the same time, because of extreme intra- and inter-population diversity, specific microbe-based interventions should be population-specific, administered only after extensive research and analysis.

Most generally, all early infant care policy and practice should reflect current understandings of gut development, and the importance of perinatal vertical inheritance (Dominguez-Bello et al. 2010; Dominguez-Bello and Blaser

2015). Perinatal care policies and practices should be reexamined thoroughly and restructured to be “microbiota-friendly.” Dramatic reductions of cesarean section procedures appear warranted (Neu and Rushing 2011). Prophylactic prescription of antibiotics is to be avoided (Langdon et al. 2016; Tamburini et al. 2016). If administered, the mother or caretaker should be educated in measures to support the regeneration of microbiota (breastfeeding, maternal and infant/child diet, probiotic supplementation, and in severe cases, fecal matter transplants) (Praveen et al. 2015; Chau et al. 2015; Elias et al. 2011; Hourigan and Oliva-Hemker 2016). Pregnant women should not be treated with antibiotics if possible; rather, vaginal Lactobacillus suppositories may be prescribed in the last trimester to support decreased infection rates, healthy birth outcomes, and positive early microbial colonization patterns (Stojanović et al. 2012).

Findings from this dissertation contribute to a growing body of research showing that most household environments in wealthy, western countries are too clean (Strachan 1989; Strachan 2000; Sheikh and Strachan 2004). In this Salvadoran population, a moderate level of microbial exposure was ideal for infant microbial and immune development. Results showed that a middle-range exposure provided a middle-low range of alpha diversity, a time when the vertically transmitted microbiota are taking residence and creating a foundation within the infant intestinal microbiome. As an infant grows, this same moderate environmental exposure, provided an increased environmental exposure, while not overwhelming or depriving this developing internal ecosystem. If future research confirms these findings, it could be recommended that public health officials in El Salvador and elsewhere describe ideal household parameters that support the early development of the infant gut microbiome in each context.

Breastfeeding has long been a global health mandate. This study and other microbiomics research should serve to support current reasoning (Armstrong et al. 2002; Labbok et al. 2004); Trevathan 1984; Trevathan and McKenna 1994; WHO 2003) and offer new motives for health care practitioners and institutions, employers, communities and families to encourage and support breastfeeding (Jost et al. 2014; Guaraldi and Salvatori 2012). Maternal lactation educational programs should be instituted both in maternity wards and local health clinics (Bernaix et al. 2008; Schy et al. 1996). Pregnant women should be taught about the benefits of colostrum and sustained, exclusive breastfeeding as well as breast pumping (Chantry 2011; Scott and Binns 1999). Infants should be kept with mothers following birth, and immediately taught to initiate breastfeeding (ibid). Intensive care unit (NICU) infants should be breastfed when possible (Hartz et al. 2015).

The Salvadoran ministry of health should have jurisdiction to oversee maternal-infant health programs at large-scale companies in El Salvador. All employers, particularly foreign investment companies, should institute living wages for women with paid maternal leave for a minimum of 3 months, and support for breastfeeding and daycare for a minimum of 6 months (Baker and Milligan 2008; Kramer et al. 2001). Flexible work hours and part-time work options should be offered to mothers with infants under 6 months (Witters-Green 2003). Mothers should receive paid family leave for infant illness (Glass and Estes 1997). Factories that employ women should have breast-feeding and pumping programs (Cohen and Mrtek 1994; Ortiz et al. 2004). These programs should offer mothers multiple breaks throughout the day for milk-pumping and provide adequate milk storage facilities. This practice This practice was at least instituted in one factory, as one study participant benefited from three, 30 minute breaks each work day to pump breastmilk, which was properly stored for her. This mother was successfully able to continue breast feeding her infant through the sixth month.

Programs and policy changes such as those recommended in the above section, are difficult to implement and enforce. It requires the marshalling of government support and resources, which few countries practice. A success story is provided by the Norwegian government, where many of the above policies have been implemented (Alvarez 2003). Policies protecting breast-feeding women's needs have been in place since the 1970s, approximately 97% of women breast feed when leaving the hospital, and 80% are breast feeding at 6 months. Government family policies play an important role in enabling women to achieve these breast-feeding rates. In Norway maternity leave is 42 weeks with full pay or 52 weeks with 80% of salary; flexible part-time is available for women from 2 months after giving birth with income supplemented from maternity benefits; after returning to work, women are entitled to 1- to 1.5-hour breaks to return home to breast feed, or to have the child brought to work (“GBP” 1998). Such policies greatly enhance the health and well-being of infants, mothers and families. Exclusive breastfeeding supports the growth and development of infants, prevents infant morbidity and mortality, encourages bonding between mother and infant, supports a more rapid weight loss and health of mother

("WHO 2017), and more recently has been shown to improve infant cognition (Jedrychowski et al. 2012) and adult functioning, health, and employment (Martorell 2010). Extensive research has analyzed the benefits of specific nutritional interventions and can support the development of a locally relevant and effective intervention program (Adair et al. 2013; Bhutta et al. 2008; Martorell et al. 2010). El Salvador is well-positioned to be a leader in guiding maternal-infant policies that support the health of future generations, modeling these policies and interventions for the rest of Central America, other developing populations and even many developed nations.

If implemented, these changes could dramatically impact the health and lives of future generations in El Salvador. Unfortunately, larger political-structural issues in this country require attention and resolution for the health and well-being of future generations, including socio-economic instability and among the highest rates of violence in the world. The Salvadoran government has approached the problem of gang violence from a crime and security angle, with a 'mano dura' or 'tough handed' tactic, focusing exclusively on law-enforcement (Abrahamsen et al. 2009). While justice and police have an important role to play, these tactics are repressive and counter-productive, as they only incite more violence and avoid the root causes of gang-related violence and instability (ibid).

Government policy should redirect attentions from the criminalization of poverty to consider structural issues that promote gang recruitment and social instability. A growing body of research suggests that violence and poverty are not directly correlated: even as Central American countries overcome extreme poverty, some become more violent. In the case of El Salvador, poverty and lack of economic opportunity certainly play parts in the recruitment of gangs, however the criminalization of poverty only serves to polarize the issue. For effective change, the Salvadoran government needs to consider violence and social instability as threats to public health. The underlying issues need to be comprehensively understood and addressed with well-targeted solutions.

Underlying risk factors for why young men get involved with gangs should be assessed, and interventions developed for young males between 10-30 years old (Bruneau, Dammert, and Skinner 2011). The influx of multinational corporations and a decrease in subsistence farming has left few employment and educational opportunities for young men. Treatment plans involving quality education, skill development and job opportunities should be priorities for these programs. Workforce development programs that are targeted at young men with previous gang involvement, should take holistic perspectives, focusing not only on skill training, but also on therapy and trauma counselling (Moser and McIlwaine 2006). Families and communities should be included in these training and recovery programs. Prevention is also important, where affordable and quality childcare, as well as community support for young families are key to overcoming youth violence and gang recruitment.

For residents of Joya de Cerén and most other regions of El Salvador, violence has precipitously eroded trust and quality of life among neighbors and families. This has also served to further strain childcare networks and put increased demands on young families. The consequences are wide-reaching, and felt daily by all residents to a certain degree: from deep fear of safety, to the loss of a loved one. The assassination of Ana, Joya de Cerén's health promotor, deeply shook the community. One study participant commented to me after Ana's death that "If they killed her in her *home*, none of us are safe!" Another lamented "we feel terrible without Nina Ana, there is no one else here, I can say that we feel very alone without her." Only with a stable, safe environment can issues of infant health and development be addressed. Until then, positive effects from interventions will only be short-lived.

Future research

Until now, developmentally-inclined microbiomics research has focused nearly exclusively on understanding the effects of birth mode, early feeding practices and antibiotics on microbial assembly (Dominguez-Bello et al. 2010; Bäckhed et al. 2015; Palmer et al. 2007; Guaraldi and Salvatori 2012; Jost et al. 2014; Ajslev et al. 2011; Blaser 2014; Bokulich et al. 2016; Cho et al. 2012). Future research should consider integrated, multi-level frameworks such as developmental ecology for exploring infant microbial ontogeny (Benezraa et al. 2012; Sheets and Worthman 2014; Thompson 2012; Thompson et al. 2015), even in previously studied European and US populations. Cross-cultural application of tools such as the HMEI scale may provide comparative models for understanding how local human ecologies shape microbial diversity, albeit with locally relevant variables. Moreover, socio-political and economic factors are rarely considered in microbiomics research, even though few populations in the world are not affected by transformations in integrated globalized economies. Maternal labor patterns, for example, are rarely included into research agendas, and yet they were found to have a profound effect on infant microbial development, health and growth. Without a complex and integrated model by which to investigate the context of early development, the results of microbiomics research are only marginally applicable. Long-term birth cohort studies that identify both shared and distinctive features of microbial community development, within and across populations, can support understandings of "healthy" development and relationships to health status. With an integrated, multi-level research agenda, discoveries of microbial variation can be identified and the construction of population-specific "healthy" parameters of infant microbial development established. Deviations from these established parameters can support risk assessment and development of microbiota-directed therapeutic interventions and other approaches for up-stream disease prevention.

Future research can incorporate a cross-cultural analysis, comparing this dataset to that of other developed and developing countries. Understandings of both the intra- and then inter-population variation can support the modeling of time-dependent vertical and horizontal exposures, and ideal windows of microbial development.

Conclusion

This dissertation has explored the ways in which vertical (birth mode and early feeding) and horizontal (socio-political and ecological processes) microbial transfers are effected or obstructed during ontogeny of microbiota and production of individual microbial variation, and subsequent effects on infant health and growth. These chapters identified concrete connections among macro patterns of labor markets, demographics, and the micro-biota of the infant intestinal tract, with significant biological and health consequences. It has exemplified the value of incorporating a multi-level, host-microbe-ecology integrative model that fruitfully can be used to study diverse populations around the world. Findings in each chapter of this dissertation revealed important correlates of cues to microbial ontogeny in general, as well as many relevant to this setting in particular. Sections of this dissertation will be translated and submitted to the aforementioned Salvadoran institutions such that policy recommendations from this study can be discussed and implemented. This dissertation adds new depth to extensive existing literature showing not only that context matters during development, but also that early environments can have long-term consequences on human health, growth, and biology, with possible intergenerational implications (Kuzawa 2008; Kuzawa and Quinn 2009; Kuzawa et al. 2007; Thompson 2012). If our microbial inheritance is to be preserved and sustained, biomedical and public health programs globally will need to be reoriented. In accord with other lines of work in maternal-child health (Black and Dewey 2014; Black et al. 2015; Grantham-McGregor et al. 2007), the present findings suggest that multi-national corporations and other labor markets must support working mothers during the first years following the birth of their children.

Furthermore, this dissertation has provided novel analytic methods to detect patterns that affect infant microbial development and identify intra-population parameters of age-dependent "healthy" development, in the hopes that research teams, corporations and governments begin to see the inter-dependent nature of cultures, human populations, our co-evolved resident microbes and our broader ecosystems. The broadest intention of this dissertation was to develop tools, methods, biomarkers and frameworks that will support the operationalization of interventions and policy that can help alleviate suffering and enhance well-being in this, and many other populations worldwide.

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