EARLY LIFE EFFECTS OF A DUAL BURDEN ENVIRONMENT:
CHILDHOOD INTESTINAL HEALTH AND IMMUNE FUNCTION IN GALÁPAGOS, ECUADOR

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ABSTRACT

Kelly Marie Houck: Early Life Effects of a Dual Burden Environment: Childhood Intestinal Health and Immune Function in Galápagos, Ecuador
(Under the direction of Amanda Thompson and Mark Sorensen)

Early life pathogenic and nutritional environments impact health over the life course by training the immune system to adapt to local microbial conditions and developing metabolic trajectories based on resource availability. Exposure to environmental microbes during childhood, common throughout evolutionary history, can provide immunoregulatory properties that strengthen the immune system’s ability to resolve inflammation. In populations with childhood undernutrition, pathogenic exposures due to unsanitary living conditions can cause chronic intestinal inflammation. This condition, known as environmental enteric dysfunction, allows for microbes to enter the blood causing endotoxemia and systemic infection. Chronic immunostimulation during childhood is energetically demanding and often results in growth deficits.

This dissertation uses the emerging field of the gut microbiome as pathway to investigate the early life effects of overnutrition and poor water quality on childhood intestinal health and immune function in Galápagos, Ecuador. Residents of San Cristóbal are unfortunately experiencing a dual burden of both increasing rates of obesity, coupled with persistent rates of infectious disease. Data was collected from 169 children aged two to ten and their 119 mothers. Interviews obtained information concerning household water use and sanitation practices, and children’s hygiene behaviors, illness histories and diets. Household water samples were collected to quantify fecal pathogens. Anthropometric assessments provided indicators of nutritional
status. Blood spots were measured for immune biomarkers and fecal samples were collected to examine gut microbial compositions.

Novel hypotheses are tested for the dual burden environment that examine the relationship between pathogenic and obesogenic factors on inflammation, endotoxemia and gut microbial composition, and provide insight into the early life health impacts of the dual burden environment on childhood intestinal health and immune function. The significance of this research is that even in the context of a pro-inflammatory state, driven by overweight and obesity, early life exposure to *Escherichia coli* contaminated water, which does not result in diarrhea, can provide an immunoregulatory effect among children in Galápagos. Identifying gut microbial symbiosis as a possible mechanism underlying this protective effect is an original contribution to the evolutionary “old friends” hypothesis and is of particular importance to public health research on environmental enteric dysfunction.
To Mom, Dad, Héctor and Luka.

I hope to make you proud.
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<tr>
<td>AHA</td>
<td>American Heart Association</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>CDC</td>
<td>Center for Disease Control</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>DOHaD</td>
<td>Developmental origins of health and disease</td>
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<td>EED</td>
<td>Environmental enteric dysfunction</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EndoCAb</td>
<td>Endotoxin core antibodies</td>
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<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin-G</td>
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<tr>
<td>kg</td>
<td>Kilograms</td>
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<tr>
<td>L</td>
<td>Liter</td>
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<tr>
<td>L:M</td>
<td>Lactulose: mannitol</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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<td>m</td>
<td>Meters</td>
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<tr>
<td>mg</td>
<td>Milligrams</td>
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<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>MPN</td>
<td>Most probable number</td>
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<tr>
<td>MU</td>
<td>Median units</td>
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<tr>
<td>n</td>
<td>Total number of observations</td>
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<td>OTUs</td>
<td>Operational taxonomic units</td>
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<tr>
<td>ROC</td>
<td>Receiver operator characteristic</td>
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<tr>
<td>SCFA</td>
<td>Short-chain fatty-acid</td>
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<td>SD</td>
<td>Standard deviation</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>SE</td>
<td>Standard error</td>
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<tr>
<td>SES</td>
<td>Socio-economic status</td>
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<td>Th</td>
<td>Helper T cell</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>WASH</td>
<td>Water, sanitation and hygiene</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>UNC</td>
<td>University of North Carolina</td>
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<td>μL</td>
<td>Microliter</td>
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CHAPTER 1. INTRODUCTION

Exponential growth of the tourism industry over the past several decades has ignited economic and urban development and mass migration to the three major populated islands of the Galápagos Archipelago (Epler 2007; Taylor et al. 2009), causing rapid changes to human health and the environment (Walsh et al. 2010). While the islands are famous for research on the biodiversity of natural systems and the social impacts of human-environment interactions (Santander et al. 2008), much less is known about how these changes are influencing human health and nutrition of residents. Emerging population health research in the Galápagos identifies the lack of clean water, poor sanitation and limited access to medical services as major health concerns (Page et al. 2013; Walsh et al. 2010). A 2013 water quality study on the island of San Cristóbal reported high levels of Escherichia coli in the municipal water network, with 72% of point-of-use samples at high health risk and additional 18% at very high risk (Gerhard et al. 2016). Common illnesses are typical of populations with poor environmental quality, such as gastrointestinal, respiratory, urinary and skin infections (CGREG 2010a; Walsh et al. 2010); yet there are also concern for growing rates of obesity and diabetes (Page et al. 2013; Tufton and Chowdhury 2015). The lack of access to fresh produce and the reliance on processed foods shipped from the mainland further limit dietary diversity and nutritional quality. A recent study on the island of Isabella reflects a pattern seen elsewhere in Ecuador (Freire et al. 2014b; Houck et al. 2013), where overweight mothers are found in the same households as both underweight and overweight children (Waldrop et al. 2016). This suggests that Galápagos and Ecuador, like many developing countries are now undergoing nutrition transitions with a shift to calorically-
dense diets, high in sugars and fats and low in fiber (Popkin et al. 2012). Ecuador’s 2012 National Health and Nutrition Survey reported that Galápagos children have the highest rates of overweight and obesity in the entire country (Freire et al. 2014a). A better understanding of the health impact of living in a dual burden environment, where higher infectious disease burdens are coupled with increasing rates of obesity and cardio-metabolic disorders, is crucial to improve the lives of children from Galápagos, Ecuador.

Adverse environmental conditions and events early in life have consequences for adult health (e.g. Barker 1994). The developmental origins of health and disease (DOHaD) framework investigates the health impact of a mismatch between changing nutritional and disease environments from early to later life (Adair and Prentice 2004; Gluckman and Hanson 2006). Much attention is focused on the link between prenatal undernutrition and postnatal overnutrition with later risk of cardio-metabolic disease (Adair and Cole 2003; Gluckman et al. 2007). Studies on the development of the immune system suggest that inflammation is a key mechanism linking childhood health and growth with adult risk of cardio-metabolic disease (Danesh et al. 2004; Tzoulaki et al. 2008). Infants are born immunologically naïve, and evolutionary theory suggests that early life disease exposure influences the development of immune function and inflammatory trajectories throughout the life course. The “old friends” mechanism suggests that modern lifestyles and improved living conditions in high income countries limit exposure to immunoregulatory pathogens common throughout human evolution, causing immunodysregulation and higher rates of asthma, allergies and other autoimmune disorders (Rook et al. 2017). Conversely, exposure to these pathogens will provide immunoregulatory benefits and strengthen anti-inflammatory networks (Yazdanbakhsh et al. 2002). Only a few studies have investigated the impacts of early environments on inflammation and immune health.
in children from non-Western populations with low resources (Blackwell et al. 2010; McDade et al. 2005; McDade 2005; Nazmi et al. 2009) and little is known about the early life consequences of a dual burden environments on immune regulation.

The energetic costs of maintaining robust immune function during childhood in environments with heavy pathogen burdens comes at the expense of linear growth restrictions, often resulting in stunting (McDade et al. 2008; Stephensen 1999). Evolutionary life history theory provides a framework to study human variation in terms of energetic investments in life history strategies or events throughout the life course, such as birth, growth, maturation, and pregnancy, which have been shaped by natural selection (Hill 1993). Studies modeling energetic life history tradeoffs between immunocompetence (normal immune response to pathogens) and growth during childhood have traditionally focused on populations with scarce food resources. In malnourished environments, chronic intestinal inflammation and systemic immune activation caused by repeated pathogen exposure due to poor sanitation contributes childhood stunting (Campbell et al. 2003a; Humphrey 2009; Prendergast et al. 2014). This is known as environmental enteric dysfunction (environmental enteropathy), a subclinical condition characterized by local inflammation and poor intestinal barrier function allowing for the translocation of microbes into the blood stream resulting in endotoxemia (Syed et al. 2016). This chronic state of immune dysfunction can divert energetic resources from normal growth and development during childhood in populations experiencing undernutrition. However, in countries already plagued by overnutrition, excess adiposity also induces an inflammatory response (Hotamisligil 2006) and some have argued that dietary-induced gut inflammation creates a feedback loop resulting in obesity (Cani et al. 2007; Ding and Lund 2011; Zhang et al. 2008). Under the traditional life history framework described above, we expect to find little evidence of
growth restrictions in high food resource environments during childhood because the amount of energetic resources available would cover the cost of pathogenic immunocompetence (McDade 2003). In dual burden environment, concurrent pathogenic and obesogenic exposures may further contribute to gut immunodysregulation, creating a new synergism between overnutrition and infection contributing to environmental enteric dysfunction.

The emerging field of gut immunology has the potential to contribute to evolutionary theory and broader health concerns by providing a new pathway, the gut microbiome, to examine the effects of the pathogenic, nutritional and social environments on human physiology and consequently variation in human growth and development. Over the course of human evolution, gut microbiota have developed a symbiotic relationship with their host, and their bacterial metabolites play a vital role in regulating metabolic and immune function (O’Hara and Shanahan 2006). Pathogens and dietary patterns can influence the composition of microbiota and consequently alter the production of metabolites causing gut immunodysregulation (De Filippo et al. 2010; Kau et al. 2011). Although changes to the gut microbiome caused by habitual ingestion of fecal pathogens in contaminated water and food have been hypothesized to underlie the etiology of environmental enteric dysfunction (Brown et al. 2015; Kau et al. 2011), its role remains untested in human populations.

This project modifies versions of life history theory and the DOHaD framework to fit the dual burden environment, accounting for the traditional pathogen-induced immunocompetence and incorporating obesity-induced immunostimulation (Figure 1.1). These evolutionary anthropological frameworks are used as the foundation for this dissertation examining the effects of early life environments on childhood gut and immune health in Galápagos, Ecuador. New predictions are tested about immunoregulation in the dual burden environment through the
innovative use of gut microbial analysis, as an indicator of intestinal health, in relation to systemic inflammation and endotoxemia associated with environmental enteric disorder.

1.1. Paper 1- Measuring Chronic Low-Grade Inflammation

The objective of Paper 1 is methodological in disentangling the use of C-reactive protein (CRP) as a marker of inflammation in mothers and children living in a dual burden environment. CRP is both reactive to infection causing acute, high elevations and obesity, resulting in chronic, low-grade elevations. Elevated ranges commonly used to indicate chronic, low-grade inflammation as cardiovascular disease risk in populations with low infectious disease burdens, may be problematic due to infection-induced elevations in high pathogenic environments. Several methods are tested for measuring low-grade, chronic inflammation using cross-sectional and longitudinal data, to determine which is the most discriminating in predicting obesity using specificity and sensitivity analyses. The findings provide methods for estimating chronic, low grade inflammation in a dual burden population, along with practical health statistics for the study population.

1.2. Paper 2- E. coli Exposure and Immune Function

The aim of Paper 2 is to determine the impact of early life fecal pathogen exposure from contaminated household (non-drinking) tap water on inflammation and endotoxemia in children. Studies on environmental enteric dysfunction suggest that high levels of Escherichia coli, an indicator of fecal contamination, is associated with chronic immunostimulation (Humphrey 2009); however, using the “old friends” theory I hypothesize that habitual exposure that does not result in acute infection may lower immune levels. Regression models are used to test this hypothesis that E. coli exposure provides immunoregulatory benefits to inflammation and endotoxemia, separately. This study contributes to the public health literature on water,
sanitation and hygiene studies (WASH) and evolutionary theory by providing novel evidence of an early life protective effect of exposure to fecal contamination on immunostimulation among children living in a dual burden environment.

1.3. **Paper 3- Gut Microbiota Mediate Immunostimulation**

The purpose of Paper 3 is to determine whether the gut microbiome is a mechanism underlying the immunoregulatory effect of exogenous fecal pathogen exposure among children from Galápagos. First, gut microbial taxa that are significantly influenced by fecal pathogen exposure in household tap water are identified. The effects of those taxa on levels of immunostimulation in children are determined. Specific gut microbiota are determined to be responsible for the protective effect on inflammation and endotoxemia if fecal pathogen exposures provide gut immune symbiosis, as opposed to causing dysbiosis. These results identify several candidate gut microbial taxa as key modifiers of the immunoregulatory effects of early life fecal pathogen exposure in children from Galápagos, Ecuador.
Evidence from Developing Countries

- High Disease Loads

Evidence from Western Countries

- High-Fat/ Low-Fiber Diets

Gut Immune Dysregulation: Pro-inflammatory State

- Environmental Enteric Dysfunction
- Inflammatory Disorders
- Overweight/ Obesity

Overnutrition and Infection in Dual Burden Environments

*Other exposures outside the scope of this project: genetics, maternal transfer, psychosocial stress

Figure 1.1 Conceptual model of the health consequences of the dual burden environment
CHAPTER 2. LIFE, HEALTH AND NUTRITION IN GALÁPAGOS, ECUADOR

2.1. Ecuadorian Health Landscape

Urbanization, industrialization and globalization are key processes shaping the disease landscape of the second epidemiological transition in affluent developed countries where declines in infectious diseases and malnutrition are taken over by chronic, non-infectious degenerative disease (Armelagos and Harper 2010; Barrett et al. 1998; Omran 2005). Urbanization has extensively transformed human health, not only in changing social structure, behavior and lifestyles, but also in the form of exposures to new stresses (Schell 1997) and protections from others (McMichael 2000; Satterthwaite 1993). Improvements in nutrition (McKeown 1976), public health, sanitation and living conditions (Woods 1990) are regarded as being primarily responsible for the second transition in the high income countries. However in many low and middle income countries, social inequality, especially in urban areas, results in an overlap of both infectious and chronic disease due to the lack of adequate drinking water, sanitation infrastructures and medical services, along with changing dietary patterns and physical activity levels. The introduction and reliance on new food products in developing markets through international trade and globalizing forces is particularly concentrated in urbanized areas (Popkin and Gordon-Larsen 2004). This transition is responsible for increasing rates of overweight and obesity in the developing countries and has created a dual burden situation where stunting and obesity are associated at the individual and household levels (Doak et al. 2004; Sawaya et al. 1998).
Ecuador is currently experiencing this dual burden of chronic and infectious diseases (Waters 2006). Ecuador’s Living Standards Survey in 2014 reports that 78% of households have access to the public water networks, though only 60% of bathrooms are connected to the public sewage system (INEC 2014). Although these indicators of basic household sanitation needs have been steadily improving since 1999, health effects of are still evident among children. Roughly 25% of children under five experienced acute diarrheal disease and 46% had acute respiratory infection at the time of study (INEC 2014), which may be due to increasing levels of air pollution (PAHO 2012). Infectious diseases, such as gastrointestinal infections, influenza and pneumonia are among the top causes of death in Ecuadorian children under five (PAHO 2010). Poor hygiene and inadequate sanitation infrastructures are major health risks among children who are more susceptible to pathogens due to their developing immune system. However, more research is need to better understand how household sanitation and hygiene deficits impact childhood health and risk of infection in Ecuador.

Despite achieving middle income country status, chronic childhood malnutrition in Ecuador is a major health concern and is strongly associated with socio-economic disparities, lifestyle differences between ethnicities, and the urban-rural divide (Larrea and Freire 2002; Walker 2007; Waters 2006). In 2012, approximately 25% of Ecuadorian children under the age of five were stunted, indicating a state of chronic malnutrition, which has dropped from 34% in 2004 (Freire et al. 2013). However, Ecuador is also undergoing a nutrition transition with a shift from low calorie plant-based foods to a high calorie diet rich in refined carbohydrates, fats and sugars, resulting in increasing rates of obesity and cardio-metabolic disorders (Bernstein 2008). Freire and colleagues (2014b) found that 13% of households nationwide in Ecuador had overweight or obese mothers and stunted children, and at the individual level, 3% of children
aged five to ten were overweight or obese and stunted. The health effects of this transition in Ecuador are evident in the top five causes of death for adults over 45 years according the Pan American Health Organization, of which three are attributable to cardio-metabolic problems (PAHO 2010). Since levels of economic development and urbanization are highly influential in shaping dietary patterns associated with both chronic childhood malnutrition and overweight and obesity in Ecuador, nutritional status is a vital component of the disease ecology of Ecuador.

Rapid economic and urban development driven by the tourism industry has transformed health and nutrition on the Galápagos Islands (Walsh et al. 2010). Over the past several decades the population has quadrupled (INEC 2010), increasing pressure on the islands’ water and sanitation infrastructures. With a heavy reliance on processed foods shipped from the mainland, residents of Galápagos currently have the highest rates of overweight and obesity in all of Ecuador (Freire et al. 2014a; Page et al. 2013). High levels of infectious disease, due to poor water quality and inadequate sanitation, are found alongside increasing rates of chronic conditions such as hypertension and diabetes (CGREG 2010b; Gerhard et al. 2016). The Galápagos Islands provide a unique research setting to investigate the health consequences of a dual burden environment.

2.2. Tourism, Economic Growth and Urban Development on the Galápagos Islands

The history of human populations on the uninhabited islands began with early visitors including whalers, pirates and naturalists (Oxford and Watkins 2009). These groups were followed by several failed attempts at colonization, the development of a profitable agricultural plantation, prisoner camps, and an American military base during WII (Astudillo 2017; Oxford and Watkins 2009). Attempts to conserve the unique biological and ecological diversity of the islands began in the 1950s when the Charles Darwin Foundation was founded to promote
conservation and research (Epler 2007). Meanwhile the Ecuadorian government established 97% of the undeveloped land on the islands as the Galápagos National Park (Walsh and Mena 2013). At that time, the four populated islands of San Cristóbal, Santa Cruz, Isabella and Floreana had limited infrastructure with residents working in agriculture and fishing. The islands became a United Nations Educational, Scientific and Cultural Organization (UNESCO) World Heritage Site in the 1978 (Walsh and Mena 2013). Although the tourism industry began much earlier, it was not until the late 1980s with a fundamental shift from boat-based tourism to land accommodations that the islands’ residents started engaging in the economic development of tourism (Epler 2007). Mainland Ecuadorians also began to migrate to Galápagos seeking opportunity in the profitable tourism industry, especially since Ecuador’s economy was suffering due to the devaluation of oil prices (Epler 2007).

Since that time, the population has expanded from around 6,000 in the early 1980s to over 25,000 in 2010 (INEC 2010), with over 200,000 visitor annually (Schep et al. 2014). Although the Ecuador government has instated laws to regulate immigration to the island, illegal residency is still a problem (Walsh and Mena 2013). The tourism industry in Galápagos is growing rapidly with a 78% surge in gross domestic product during the first half of the 2000s (Taylor et al. 2009). According the Galápagos Conservatory Organization, tourism contributes $418 million US dollars to the Ecuadorian economy with less than 7% returning to the islands’ economy. In 2009, the provincial government of Galápagos received a total of 14.5 million dollars, with over 94% being invested in the construction of public roads and buildings (CGREG 2010b). However, living conditions have not improved. Less than 1% was spent on municipal drinking water and sewer systems, which was below the 3% investment in urban beautification projects. The impact of population growth and economic and urban development driven by
tourism has undoubtedly change the ecological and social environments of the islands, increasing pressure on the limited infrastructures and resources (Walsh and Mena 2013).

2.3. People of San Cristóbal

The 2010 Ecuadorian census reports that 7,475 people live in the canton of San Cristóbal, which is the second largest populated canton following Santa Cruz (INEC 2010), and represents 30% of the total population for Galápagos (Figure 2.1) (CGREG and INEC 2010). The island has experienced high population growth over the past several decades, mainly from migration from the mainland. In 2010, approximately 66% of the islands’ population were migrants, mostly from mainland Ecuador moving to join family on the island or for economic opportunities (CGREG and INEC 2010). Most Galápagos residents live in concentrated urban areas consisting of 3% of the islands’ land, since the majority is protected national park space. On San Cristóbal, people live in the urban port town and surrounding neighborhoods of Puerto Baquerizo Moreno (Figure 2.2), which comprises 82% of the canton of San Cristóbal (CGREG and INEC 2010). Thus, the town provides a good representation of the survey area for this dissertation project.

The dominant ethnicity in San Cristóbal is mestizo (which represents a mix between Indigenous Ecuadorian and Spanish descent) at 81%, followed by indigenous ethnicities from the mainland at 9%, European ancestry at 6% and Afro-Ecuadorian ethnicity at 4% (CGREG and INEC 2010). As in the rest of Ecuador, Spanish is the most common spoken language followed by indigenous languages.

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1 Inhabitants of the small island of Floreana and rural residence of the highland town of El Progresso on San Cristóbal are included in the reported statistics for San Cristóbal Canton.
The average age of women is 45 years and women were considered the head-of-household in 16% of homes on the island (CGREG and INEC 2010). In terms of demographic dependence, which provides a measure accounting for the proportion of active-working age to dependent children under 15 or adults over 65 years, 54% of residents are dependent on working age residents. Roughly 64% of adults are either married or living in a common law marriage, while the divorce or separation rate in San Cristóbal is low at 4% (CGREG and INEC 2010).

Education and literacy in Galápagos are high. Around 96% of children attend primary school and 90% attend secondary school, while 35% of children under five years attended preschool (CGREG and INEC 2010). There are four primary schools on the island, two are public and two private. Universidad de San Francisco de Quito has a campus on the island and a well-attended English language program. Under 3% of children cannot read or write by the age of 15. Among adults, 47% finished secondary school while 21% finished higher levels of education (CGREG and INEC 2010).

Roughly 68% of residents are of working age in San Cristóbal, including 62% of women, with an unemployment rate of just above 2% (CGREG and INEC 2010). A high percentage of workers are engaged in some form of public administration at 23%, which is much higher compared to other islands because Puerto Baquerizo Moreno is the capital town of the province (CGREG and INEC 2010). This is followed by commerce at 12%, transportation at 11%, and then tourism and agriculture or farming at around 8% each (CGREG and INEC 2010). In a somewhat conflicting report on 2009 data from the employment management system in Galápagos, around 21% of jobs on San Cristóbal are in public administration, with an additional 21% in the tourism industry (CGREG 2010b). Approximately 10% of jobs are in agriculture and less than 1% are from the fishing industry. Home businesses are also quite common in
Galápagos, with most participating in vending food and other goods, or small restaurants and hotels (CGREG and INEC 2010).

2.4. Childhood Health and Nutritional Status

Childhood health and nutritional status are important indicators of overall population health, and are of particular interest given the dual burden environment of Galápagos since children are more susceptible to changing nutritional and pathogenic environments (DeBoer et al. 2012). Only 38% of children under five years of age receive annual health checkup on the island (CGREG and INEC 2010), which may be due to the lack of trust mothers have with the public hospital and staff (Page et al. 2013). However, vaccination rates are high with 100% of children receiving tuberculosis BCG, 96% receiving poliomyelitis OPV and 92% receiving the measles, mumps, rubella (MMR) vaccine. During the time of the Galápagos Living Conditions Survey, around 8% of mothers reported that their children under five had acute diarrheal disease, all of whom were under doctor’s care and the majority were taking medication or oral-rehydration fluids (CGREG and INEC 2010). The rate of diarrheal infection on the island was much lower than the national average, yet the rate of respiratory infections was equivalent (INEC 2014). Again, the majority of children with symptoms were receiving medication and medical attention. Although estimates for children are unknown, around 64% of the total island population had received deworming medication, yet it is unclear whether this reflects preventive or active treatments of infection (CGREG and INEC 2010).

According to Ecuador’s National Health and Nutrition Survey in 2012 (ENSANUT), children living in Galápagos have the lowest rates undernutrition and highest rates of overnutrition compared to all other regions in Ecuador (Freire et al. 2014a). Among Galápagos children under five, only 11% are stunted, compared to 25% on the national level (Figure 2.3).
Galápagos was also the only province were no child exhibits wasting, a short-term indicator of severe weight loss, compared to 4% of all children in Ecuador. At the national level, childhood stunting is strongly linked to poverty, limited maternal education and indigenous ethnicity (Freire et al. 2014a).

Conversely, rates of overweight and obesity are high. Approximately 13% of Galápagos children under five are either overweight or obese, compared to 9% in all of Ecuador (Figure 2.3) (Freire et al. 2014a). Similar to low income countries, there is a positive relationship between obesity and income quintiles at the national level, except for the highest quintile that has the lowest rate. Yet more like a pattern seen in higher income countries, obesity is inversely related to mother’s education, again with the exception of the highest level of education exhibiting the highest obesity rate (Freire et al. 2014a). Research at the national level is needed to understand the relationship between mother’s education and household income quintiles and their impact on childhood obesity.

The divergence in rates of overnutrition and stunting between Galápagos and all of Ecuador are even more extreme in children aged five to ten (Figure 2.3). The prevalence of overweight or obesity is almost three time greater among Galápagos children and the prevalence of stunting is almost four times lower (Freire et al. 2014a). The highest rates of obesity in this age group at the national level are found among the ethnic group including mestiza and European ancestry (Freire et al. 2014a). It will be important to investigate what specific dietary and activity related factors, among other socio-demographic and environmental issues, are contributing to these extreme rates of overnutrition in Galápagos.
2.5. Adult Nutritional Status and Health

Overnutrition among the adult population in Galápagos is a growing concern, especially in relation to chronic disease risk. Rates of overweight or obesity in Galápagos adults are the highest in the country at 76%, over 10% higher than the national prevalence (Figure 2.3) (Freire et al. 2014a). Around 66% of Ecuadorian women and 60% of Ecuadorian men are overweight or obese. Rates increase with age and are the highest among Afro-Ecuadorians and the mestiza/European ethnic groups (Freire et al. 2014a). Although declining over the past decade, cardiovascular disease remains the leading cause of death in Ecuador (WHO 2014) and, between 2005 and 2016, obesity replaced malnutrition as the number one risk factor for deaths and disability according to the Global Burden of Disease Study (2017). Rates of diabetes in Galápagos have seen a dramatic 12-fold increase, whereas hypertension has more than doubled (CGREG 2010a). Considering the high obesity rates, it is surprising that 47% of residents reported playing sports or being active for almost two hours each day (CGREG and INEC 2010). There are several public sports centers scattered throughout the island’s neighborhoods and one private gym. Due to the concentrated nature of urban development on the island, most residents walk as a primary means of transportation. A study of the mothers’ perceptions of body size on the island of Isabella found that women preferred to be smaller and wished their children were larger (Waldrop et al. 2016). A better understanding of the factors underlying high rates of obesity in Galápagos are crucial in preventing cardio-metabolic disease on the islands.

Approximately 63% of the adult population in San Cristóbal has some form of health insurance, which is comparable to the average in Galápagos (CGREG and INEC 2010). In 2009, there were two doctors for every 1,000 residents in Galápagos (CGREG 2010a) and at the time
of the dissertation study, there was one public hospital, one social security health center and one rural health center on the island.

Galápagos residents are experiencing the effects of a dual burden environment, with increasing rates of cardiovascular and metabolic disorders, coupled with infectious disease. According to a report on data from the public hospital, diarrhea and gastrointestinal infections, and urinary infections were among the top ten causes of hospital internments in 2009, among complications due to pregnancy, bone fractures and injuries, and disease of the gallbladder and appendix (CGREG 2010a). Based on weekly reports submitted to the Ministry of Public Health in Galápagos of all diseases and illnesses treated at the hospitals and public health centers in 2009, 59% of all cases on San Cristóbal were acute respiratory infections, followed by over 8% due to acute diarrhea disease, 6% from sexually transmitted diseases, 2% caused by violence or accidents, including domestic and work related issues, and less than 1% of each: hypertension, diabetes, anxiety, depression and suicide attempts (CGREG 2010a). Respiratory infections in all of Galápagos have seen a sharp rise between 2006 and 2009 with more than a 150% increase, whereas rates of diarrheal disease has shown a more steady increase of 13%. More research is needed to better understand the causes underlying increasing rates of both chronic, non-communicable and infectious diseases, and their implications for residents of Galápagos.

2.6. Diet and Food Availability

Food availability is a critical issue in Galápagos since the majority of the island is national park land and there are limited areas for agriculture and food production. Most food items are shipped from the main land by boat, which can take up to two weeks, or sometimes arrive by plane. Thus, most foods are packaged staple items and fresh produce is often limited or spoiled (Page et al. 2013). There is a weekly farmers’ market where residents from the highlands
sell produce; yet diversity, availability and pricing vary. The limited availability and consumption of fresh fruits and vegetables is evident from data on household food expenses. Monthly expenditures on foodstuffs on the island reveal that chicken is the most common purchased item, followed by bread, beef, fresh fish, rice, milk and cheese (CGREG and INEC 2010). Some researchers have suggested that there is a lack of education concerning the nutritional consequences of highly processed, low value foods that may be contributing to poor diets (Waldrop et al. 2016).

As a possible indicator of food insecurity, a 2013 report on children and adolescents in Galápagos found that 17% never eat breakfast and 20% only sometimes eat breakfast during weekdays (Granda Leon et al. 2013a). However, it is unclear whether this is a direct result of a lack of resources. Especially given than there are several social programs where families can receive fortified cereals, drinks and school lunches for their children (CGREG and INEC 2010).

2.7. Household Income, Living Conditions and Assets

Approximately 47% of households on San Cristóbal own their homes, which is the same for all of Galápagos (CGREG and INEC 2010). Although poverty is apparent on San Cristóbal, most people do not consider their household as being financially poor (CGREG and INEC 2010). A 2013 report assessing measures of poverty in all of Galápagos found that 10% of households are in extreme poverty, based on two or more unsatisfied needs, such as inadequate sanitation, overcrowding, children not attending school or high economic dependence (Granda Leon et al. 2013b). In contrast, the same study revealed that based on the extreme poverty line method, which accounts for household income per capita in relation to expenditures on food consumption, no households were below the line. Income in Galápagos is higher than on the mainland, yet the cost of living is more expensive. The top quintile for average monthly
household income in San Cristóbal is $2,610 (US dollars are the currency for Ecuador), compared to the lowest quintile at $1,230 (CGREG and INEC 2010). As food availability is sometimes limited, prices can fluctuate and the majority of household expenditures go to food and drinks, followed by public utilities and transportation.

Perception of quality of life on San Cristóbal is somewhat low. Only 15% of residents consider themselves as having a good standard life, 79% consider their lives as more or less well, and 6% consider their lives as bad (CGREG and INEC 2010). In terms of perception of neighborhood cleanliness and noise pollution, only 16% of households report a low level of cleanliness and 16% also report high levels of noise pollution (CGREG and INEC 2010). The Galápagos Living Standards Survey categorizes deficits in living arrangements based on the quality of material used to construct the home (floor, walls and roof), the space provided per person (over 3 people per bedroom was deficient), and the basic services utilized, such as electricity, municipal water and indoor toilets (CGREG and INEC 2010). Around 57% of residents on the island have adequate housing in all three categories, compared to only 41% all of Galápagos. The majority of deficits were due to the use of poor materials for household construction, followed by overcrowding and a few did not have adequate access to basic services (CGREG and INEC 2010). Over 99% of households are connected to the municipal power grid and pay around $20 per month, and 90% receive municipal trash collection. Approximately 75% of residents of San Cristóbal have indoor bathrooms with toilets connected to the municipal sewage system, and an additional 23% are connected to septic tanks (CGREG and INEC 2010). Only a few households have outdoor latrines or no toilet in the home. During the time of the dissertation study, waste water from the municipal sewage system was untreated and discarded directly into the ocean near several frequented beaches. For household cooking purposes, over
99% of residents use gas, and for heating, 56% use gas while others rely on coal, firewood, candles or fuel, spending approximately $5 per month (CGREG and INEC 2010). As almost all homes have electricity, the use of technology in Galápagos is high. The national census reports that 46% of Galápagos residence used the internet within the past six months, 54% used computers and 79% used cellular phones (INEC 2010).

2.8. Water Use and Availability

Water quality and availability are a great matter of public health concern on the islands (Page et al. 2013; Walsh et al. 2010). San Cristóbal is the only inhabited island with highland fresh water sources, which supply water for the municipal network year-around. Roughly 93% of households receive municipal piped water that is collected and stored in household cisterns, where others rely on water delivery trucks or rain water (Guyot-Tephany et al. 2013). Some researchers have suggested that free municipal water has created a paradoxical supply and demand problem. While almost all residents are connected to the system, over half report that they do not receive enough water for their household uses (Guyot-Tephany et al. 2013). During the time of this dissertation, the city had just begun to install water meters to account for and regulate household water use. Water waste is a large concern with almost half of residents with cisterns admitting that they allow them to overflow when full (Guyot-Tephany et al. 2013).

It is a common assumption on the island that the municipal water is contaminated and not suitable for drinking. City water is used for household sanitation practices, such as washing dishes and clothes, and personal hygiene behaviors, such as showering, washing hands and brushing teeth. Based on observation, the majority of households rely on purchasing bottled water for drinking. However, the Ecuador Living Conditions Survey reports that for drinking, only 44% of residence buy bottled water, 39% boil their water, 8% treat it with chlorine, 5%
have their own filters, and 4% drink their water without any treatment (CGREG and INEC 2010). On average, households in San Cristóbal spend $20 on drinking water per month. A study on perceptions of water quality found that on San Cristóbal, 71% of participants agreed that they could not use water on the island in the same ways as on the mainland, commonly citing that it was polluted or that it can cause disease, followed by viewing water as a limited resource (Guyot-Tephany et al. 2013).

During the time of this dissertation project, in August 2013, the municipality on San Cristóbal opened a new water treatment plant (personal communication with city engineers). Although the city is continuing to install a new distribution network, the treated water was delivered through the old distribution infrastructure. A recent study investigating fecal contamination of the municipal water supply before and after the new water treatment plant found significant improvements in the reduction of \textit{E. coli} and total coliforms throughout the distribution system (Gerhard et al. 2016). However, possible contamination at the point-of-use sites or through the distribution system continued to provide indicators of fecal pathogens of non-human origin.
Figure 2.1 Populated Islands of Galápagos, Ecuador
Note: Data are from the Ecuadorian 2010 Census (INEC 2010)

Figure 2.2 Neighborhoods of Puerto Baquerizo Moreno, San Cristóbal
Figure 2.3 Prevalence of stunting and overweight/obesity in Galápagos and Ecuador

Note: Data are from ENSANUT 2012 (Freire et al. 2014a).
CHAPTER 3. LITERATURE REVIEW

3.1. Dual Burden Life History Theory

In populations living in dual burden environments, both pathogenic and obesogenic factors may synergistically contribute to gut immunodysregulation. This new synergism between overnutrition and infection may be contributing to the pattern of childhood stunting and obesity afflicting rapidly developing countries. Life history tradeoff hypotheses that conceptualize diet as exclusively energy availability need to be redesigned to account for the interaction between diet, gut immunocompetence and linear growth and obesity. Traditional hypotheses suggest that in developing countries with populations experiencing high pathogen loads and low food resources, we would expect to find stunting due to the limited energetic resources that are diverted from linear growth to maintaining immune function (McDade 2003). In populations with adequate food resources, the impact on growth would be reduced since additional resources could buffer the cost of immunocompetence. However, this framework does not account for dietary quality in which high-fat, low-fiber diets, characteristic of populations undergoing nutrition transition, produce direct effects on inflammation and may cause indirect effects due gut microbial dysbiosis. This situation can possibly lead to a synergistic relationship between dietary-induced and pathogens-induced gut immunodysregulation. This research proposes that gut immunodysregulation indicated by elevated inflammation (CRP), high microbial translocation (EndoCAb), and poor gut health (dysbiosis of microbial colonies) could result in greater energetic costs of maintaining immunocompetence. In addition to linear growth restriction
caused by the increased cost of gut immunodysregulation, higher inflammation levels could also divert resources from bone growth to adiposity deposits.

This dissertation expands upon life history theory studies of childhood tradeoffs between maintenance and growth by providing a new application for the dual burden environment that 1) uses three measures of gut immune function: inflammation, microbial composition and antibody response to microbial translocation, 2) disentangles the use of CRP levels associated with acute infection and chronic low-grade inflammation using a longitudinal method, 3) models the possible synergistic effect of early life overnutrition and pathogen exposure on gut immunodysregulation, and 4) determines the roles of the gut microbial symbiosis in mediating immunoregulation due to exogenous fecal pathogen exposure on environmental enteric dysfunction (EED).

3.2. Developmental Origins of Health and Disease

The developmental origins of health and disease (DOHaD) framework uses evolutionary and ecological developmental biology to link early life nutritional environments to developmental trajectories leading to later adult risk of cardio-metabolic diseases (Barker 1994). Drawing on the concept of fetal origins of developmental plasticity, Kuzawa and Adair (2004) suggest that a mismatch occurs between programming for poor nutritional environments in early life and later experiencing nutritional abundance, increasing risk for cardiovascular disease in adulthood. Gluckman and colleagues (2007) use the mismatch concept and propose that predictive adaptive responses use epigenetic changes in early life to set developmental trajectories, which allows for a range of phenotypic diversity from a single genotype. The foundational principle of DOHaD is that early life nutritional and energetic environments have lasting impacts on adult risk of cardio-metabolic diseases. This is of particular importance in
dual burden environments, since the impact on later life effects of simultaneous mismatched early life environments between infections resulting in programing for nutritional stress and overnutrition programing for resource abundance, are unknown. The DOHaD framework provides the foundation of the dissertation in investigating the impact of early life nutritional and pathogenic environments on childhood health in a dual burden environment.

3.3. Early Life Infectious Disease

Growth faltering in low and middle income countries typically occurs within the first two years of life (Shrimpton et al. 2001), further indicating that the nutritional and disease environments during this time are crucial. Nutrition, including the role of macro- and micro-nutrients and the impacts to the intestinal microbiome, can significantly alter immune function (De Rosa et al. 2015). Synergistic forces between malnutrition and infection during childhood can often result in stunting (Scrimshaw and SanGiovanni 1997; Ulijaszek 1996), and are highly influenced by dietary quality and quantity, along with weaning practices. The feeding of supplementary foods during weaning often exposes infants to unclean water and is correlated with diarrheal disease in Ecuador (Brussow et al. 1992). Significant associations between stunting and both diarrheal disease and helminthic infections are evident in children from the developing countries (Martorell et al. 1975; Moore et al. 2001). Bacterial and parasitic infections have critical implications to Ecuadorian children, who by the age of two, exhibit a 90% prevalence of *Escherichia coli* antibody titers (Brüssow et al. 1990). Solomons (1993) suggests that chronic inflammation resulting from a subclinical acute phase response, due to living in overcrowded housing and unsanitary conditions, could be responsible for stunting not directly attributed to disease or dietary quality. Improved sanitation infrastructures, such as clean drinking water, availability of household toilets and trash disposal, are associated with lower
diarrheal disease in Ecuadorian children under five years old (Brussow et al. 1995). Early life infectious disease burdens and unsanitary living conditions are significant drivers of childhood nutritional deficiencies and growth restrictions, and may set up trajectories of future conditions of nutritional stress.

3.4. Traditional Life History Theory

Life history theory provides an evolutionary framework for examining how natural selection influences energetic tradeoffs between growth, maintenance and reproduction at different life stages based on ecological conditions (Hill and Hurtado 1996; Stearns 1992). Key tradeoffs during childhood can occur between growth and maintenance of the immunocompetence (McDade 2003). Anthropological studies have found direct effects of immunostimulation on linear growth caused by non-specific inflammation among Tsimané children in the Bolivian Amazon (McDade et al. 2008) and Shuar children in Ecuador (Blackwell et al. 2010). In addition, chronic inflammation in combination with the humoral immune response, due to environmental enteropathy, were associated with reduced growth measures among infants in Gambia (Campbell et al. 2003a), Nepal (Panter-Brick et al. 2009) and Bangladesh (Mondal et al. 2012). Due to the concern of undernutrition in these populations, traditional life history theory frameworks have concentrated on energetic resource availability, which has limited our understanding of the energetic cost of immunocompetence in newly emerging dual burden environments. Investigating immunocompetence as a life history trait has also been critiqued for its use of a single measure of immune function (Long and Nanthakumar 2004). My research builds on this scholarship by examining the relationships between immunocompetence and measures of growth and body composition, relative to intestinal immune health at three different levels: 1) high-sensitivity C-reactive protein (CRP), an acute
phase protein used as an indicator of the innate, indirect response of inflammation (Du Clos 2000); 2) endotoxin core immunoglobulin-G (EndoCAb IgG) antibodies signifying endotoxemia and the humoral response to microbial translocation into the blood (Barclay 1995); and 3) the gut microbiome, as indicators of gut immune health (symbiosis) (Maslowski and Mackay 2011; Turnbaugh et al. 2006).

3.5. C-Reactive Protein and Inflammation

C-reactive protein (CRP) is an acute-phase protein stimulated by the pro-inflammatory cytokine interleukin-6 (Du Clos 2000). Acute levels indicate pathogenic infection (Pearson et al. 2003) and moderately elevated levels indicate risk factors for cardiovascular and metabolic disorders (Ridker et al. 2000). In anthropological research in developing countries, childhood CRP has been studied in association with infectious disease and malnutrition (Blackwell et al. 2009; Shell-Duncan and McDade 2004). For example, disease exposures from unsafe drinking water and measures of acculturation were associated with moderate childhood inflammation levels in an indigenous Bolivian population (McDade et al. 2005). In contrast, moderate childhood inflammation in the United States is associated with measures of adiposity, weight gains, ethnicity and socio-economic status (Dowd et al. 2010; Ford et al. 2003; Pirkola et al. 2010). In recent studies investigating environmental disease exposures related to contact with domesticated animals did not find any significant predictive effects on childhood low-grade inflammation in Western Europe (Mustonen et al. 2012) or in China (Thompson et al. 2013). Household environmental exposures such as inadequate sanitation, limited access to water and the presence of animal feces also failed to significantly influence levels of moderate inflammation among Chinese children and adolescents, where the primary driver was overweight and high waist circumference (Thompson et al. 2013). The number of siblings and infections of
Helicobacter pylori were also not predictive of inflammation levels among British children (Cook et al. 2000). These moderately elevated levels indicate risk factors for cardiovascular and metabolic disorders (Ridker et al. 2000), which are associated with obesity and not infection.

Using CRP levels to evaluate both acute inflammation due to infection or injury and chronic low-grade inflammation associated with obesity and cardiovascular disease risk is complicated. A common practice in epidemiology studies is using a cross-sectional measure of CRP and discarding high levels indicative of infection. This could be highly problematic in populations with high infectious disease burdens. It has been suggested that longitudinal measures are needed to evaluate intra-individual variability related to infectious exposures, as demonstrated in an indigenous Ecuadorian population with high variability, yet no chronic low-grade elevations (McDade et al. 2012b). This dissertation uses repeated measures CRP to explore variations due to infectious and obesogenic factors present in dual burden environments.

3.6. Environmental Enteric Dysfunction and Endotoxin Core Antibodies

Environmental enteric dysfunction is thought to be caused by chronic mucosal exposure to bacteria from fecal contaminated water and food, which induces the T-cell mediated pro-inflammatory response causing chronic gut inflammation and reduced barrier function (Campbell et al. 2003b). This results in the translocation of microbes across the intestinal barrier into the bloodstream, initiating endotoxemia and a systemic antibody immune response. Previously referred as tropical or environmental enteropathy, it is typically found in middle and low income countries where the majority of the population lives in unsanitary conditions characterized by unsafe drinking water, poor hygiene practices, and inadequate sanitation (Humphrey 2009; Neto et al. 1994). EED is marked by structural changes to intestinal villi causing poor nutrient absorption. Furthermore, the energetic costs of gut immune dysfunction can divert significant
resources from linear growth and weight gains in infants and children, and the condition is thought to contribute to chronic malnutrition and growth faltering in these environments (Humphrey 2009; Mondal et al. 2012; Panter-Brick et al. 2009).

Lipopolysaccharides (LPS) or endotoxins are components of gram negative bacteria released from the cell wall after destruction. Circulating levels in blood (endotoxemia) indicate microbial translocation due to a compromised intestinal barrier (Brenchley et al. 2006), and are highly correlated with bacterial DNA levels (Jiang et al. 2009). Endotoxin core antibodies (EndoCAb) provide a measure of the humoral immune response to LPS from four gut microbial species: *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes* and *Salmonella typhimurium* (Barclay 1995). Initial exposure to LPS activates the humoral immune response producing EndoCAbs: immunoglobulin G, M and A (IgG/M/A). EndoCAb levels are a more robust measure compared to endotoxins themselves because the antibodies remain in the blood for a longer period (Gardiner et al. 1995). Since EndoCAb varies by Ig type, IgG was chosen for this study because it is present from birth and stabilizes by age six (Barclay 1995). EndoCAb IgG has been tested in infants and children from diverse populations (Pasternak et al. 2010; Stephens et al. 2006) and has long been used as a marker of tropical and environmental enteropathy (Fagundes-Neto et al. 1984). For example, in a study of the effects of environmental enteropathy on infant growth faltering in Gambia, elevated EndoCAb IgG levels were negatively associated with linear growth and explained over 40% of growth faltering in infants under 15 months (Campbell et al. 2003a). Although it is clear that chronic immunostimulation associated with environmental enteric dysfunction impacts life history tradeoffs in linear growth in infants from poor resource setting, much less is known about its presence in children experiencing overnutrition in dual burden environments. Since chronic intestinal inflammation and impaired
intestinal barrier function are key component of inflammatory gut disorders common in high income countries (Pasternak et al. 2010), this dissertation proposes that obesogenic risk factors and high-fat, low-fiber diets typical of population experiencing nutrition transition, may contribute to elevated EndoCAb levels and endotoxemia.

3.7. **Gut Microbiota and Immune Dysregulation**

Within anthropology, limited attention has focused on the relationship between obesity and infection (Solomons 2007). However, awareness of the role of gut microbiota in immune function, obesity and chronic disorders is growing (Clemente et al. 2012). Gut microbiota produce short chain fatty acids (SCFAs) metabolites from the fermentation of dietary fiber, which are used for lipid and glucose synthesis (Maslowski and Mackay 2011). In mice, gut microbiota can regulate fat storage by the increased processing of plant polysaccharides (dietary fiber) (Bäckhed et al. 2004). The beneficial effects of SCFAs have also been demonstrated to help control appetites and regulate energy homeostasis (Byrne et al. 2015). In rural African children who typically consume a low-fat, high-fiber diet had more than double the amount of total SCFAs, indicating enhanced gut health compared to European children with a high-fat, low-fiber diet (De Filippo et al. 2010). Along with providing an important source of energy, SCFAs are ligands for G protein-coupled receptors that influence cytokine production, resolve inflammation and maintain the intestinal barrier (Maslowski and Mackay 2011). Animal studies demonstrate increased intestinal permeability in obese mice and those fed high-fat, low-fiber diets (Brun et al. 2007; Cani et al. 2007); yet, this has not been confirmed in human population (Teixeira et al. 2012).

Variations in microbial compositions and their metabolites are an important mechanism linking diet, immune function and obesity to intestinal health. When intestinal microbial colonies
are balanced, gut symbiosis allows for the proper regulation of inflammatory responses and immune homeostasis (Maslowski and Mackay 2011). Alterations to the microbiome and SCFA production associated with undernutrition may cause decreased immune responses. This can exacerbate the synergism between malnutrition and infection by increasing susceptibility to gastrointestinal infections and environmental enteric dysfunction (Kau et al. 2011). Conversely, environmental pathogens, obesity and high-fat, low-fiber diets can disrupt this balance, leading to microbial dysbiosis that causes immune dysregulation and inflammation. Due to the role of the gut microbiome in regulating immune function, it has been hypothesized that changes to the intestinal microbiota caused by environmental microbial exposures or diets may be underlying the “old friends” mechanism discussed below (Kau et al. 2011; Maslowski and Mackay 2011). This dissertation investigates the relationship between overnutrition and immunoregulation through the examination of the gut microbiome.

3.8. Early life Pathogen Exposures and Immunoregulation: the “Old Friends” Mechanism

Social and environmental factors characteristic of modern lifestyles, such as increases in sanitation practices, use of antibiotics in food sources, the hospitalization of births and vaccinations, all reduce exposure to pathogens in early life among high income countries (Bach 2002; Rook 2009). Originally called the hygiene hypothesis, the terminology has changed because the mechanism is not solely focused on hygiene. Now, the “old friends” mechanism suggests that without proper stimulation of the immune system through exposures to immunoregulatory pathogens found in soil, water and with animal contact, immune dysregulation occurs resulting in higher rates of inflammatory, allergic and autoimmune disorders (Rook et al. 2017). These “old friends”, such as helminths, *Mycobacterium tuberculosis*, *H. pylori* and environmental microbiota, increase regulatory T cells or direct the
differentiation of dendritic cells allowing them to induce regulatory T cells, which downregulate the inflammatory autoimmune responses by producing suppressive cytokines (Rook et al. 2014). Similarly, exposures to these pathogens help to regulate inflammatory pathways and cytokine profiles through the differentiation of Helper T cells (Th) into the Th1-subset, which offsets the allergic response of IgE driven by Th2-subset (Yazdanbakhsh et al. 2002).

Evidence of the “old friends” mechanism has been demonstrated in studies from the United States and Europe. Early life environmental microbial exposures associated with farms, household pets and day-care centers have shown to provide protection against allergies (Braun-Fahrländer et al. 2002; Krämer et al. 1999; Lynch et al. 2014). However, a study in the Philippines found that both early life disease episodes, as indicated by prevalence of diarrhea in the second year of life, and pathogen exposure, as indicated by the presents of animal feces in the household, significantly lowered the risk of elevated levels of inflammation in adulthood (McDade et al. 2010). These findings are significant to health research in human disease ecology since they suggest that traditional epidemiological models of environmental microbial exposures and infection may need to incorporate an evolutionary approach to better understand the impact on immune function and regulation, in addition to disease risk. Additional research is needed to measure and conceptualize early life infectious environments from other low and middle income countries with low rate of allergies, asthma and other autoimmune disorders. This project addresses this gap in the literature by researching the impact of early life pathogenic exposures on immunoregulation due to poor water quality and inadequate sanitation infrastructures in Galápagos, Ecuador.
CHAPTER 4. DISSERTATION PROJECT OVERVIEW

4.1. Pilot Project

The pilot project during field season one was carried out on the island of San Cristóbal from June to August 2013 in two phases: formative research and initial fieldwork. For the formative stage, I conducted interviews with five health workers and five mothers of children who had at least one child between the ages of two and five years old. Questions addressed health workers’ and mothers’ local perceptions of childhood illness and infections, water quality, healthy and unhealthy foods, childhood growth and obesity, and concerns regarding health research and the collection of blood and fecal samples among children. The objective of this formative research was to determine whether the study’s methods of explaining the project, obtaining informed consent, collecting blood spots and fecal samples, and interviewing mothers were cultural accepted, appropriate and feasible for the proposed study population on San Cristóbal.

For the initial fieldwork stage, I sampled 81 children aged two to ten years and interviewed their 59 mothers or caregivers. I collected detailed survey data on the child’s diet, symptom history, socio-demographics and household sanitation and water use. I conducted anthropometric assessments, performed household water quality tests of fecal pathogens, and collected blood spots for biomarker assay of inflammation and microbial translocation (endotoxemia). Fecal samples were collected to determine gut microbial composition. The objective of the initial fieldwork stage was to provide the first round of project data and conduct preliminary data explorations of the range and variation of measures used to address the project’s
larger theoretical objectives. These initial data were used to address the following specific aims of the pilot fieldwork:

Pilot Aim 1) To describe the range and variation of gut health, immune function, and adiposity and growth measures in children from the island of San Cristóbal.

Pilot Aim 2) To determine possible pathogenic, dietary, social and demographic predictors of childhood gut immunodysregulation and adiposity and growth outcomes.

Pilot Aim 3) To explore the relationship between gut immunodysregulation and adiposity and growth outcomes.

4.2. Dissertation Project

The second field season began in January 2014 and was carried out over four months. First, a follow-up study was conducted by re-visiting participants from field season one, and then, an additional 88 children were sampled along with their 60 mothers or caregivers, bringing the total to 169 children and 119 mothers. The final season fieldwork was conducted in July and August 2014 to perform the follow-up study on participants recruited during field season two. Data from all three field seasons were used to address the following dissertation project aims.

Dissertation Aim 1) To test the synergistic relationship between overnutrition and infection on gut immunodysregulation. I hypothesized that the interaction between dietary overnutrition and poor water quality was associated with higher levels of gut immunodysregulation, compared to healthy diets and poor water quality or overnutrition and safe water quality. The results of these analyses provided the foundation and were reformulated for Paper 2- E. coli Exposure and Immune Function.

Dissertation Aim 2) To determine the energetic cost of pathogenic and obesogenic immunocompetence on adiposity and childhood linear growth by testing modified LHT
hypotheses for the dual burden environment. A) I hypothesized that high levels of gut immunodysregulation was associated with high levels of adiposity and low linear growth measures, separately. B) In addition, I hypothesized that high levels of adiposity was associated with low linear growth measures. The results of these analyses were presented at the Annual Meeting for the Human Biology Association in 2015 (Houck et al. 2015).
CHAPTER 5. STUDY DESIGN, DATA COLLECTION AND MEASURES

The pilot study’s protocols, recruitment, research design and informed consent documents were based on my previously fieldwork experience with a similar project investigating childhood health in the Ecuadorian Amazon (Houck et al. 2013) and modified to the Galápagos setting based on suggestions from other University of North Carolina (UNC) researchers who have conducted fieldwork in the Galápagos in association with the Galápagos Science Center. The Galápagos Science Center is a state of the art research facility on the island of San Cristóbal, jointly partnered by UNC and Universidad de San Francisco de Quito to promote science and education. As a graduate research associate, I was able to use the center’s infrastructures to acquire appropriate permits, carry out my fieldwork and perform laboratory analyses. The center’s microbiology laboratory provided use of their equipment to conduct water quality tests and urine analyses. According to the Ecuadorian Natural Health Law (Ley Orgánica de Salud (CND 2006)), blood samples may not be taken from the country and all biomarker assays were performed at the center (Law 67, Part 2, Chapter 4, Section 80). I was loaned a microplate reader from the Amazon project and shipped it to the island to run the blood spot assays. The fecal samples were transported back to UNC and analyzed at the Microbiome Core Facility. Since participating in health research focused on mothers and children may be a personal and culturally sensitive topic, a local mother was hired as a research assistant for the project to help recruit and conduct all interviews with mothers and caregivers. All research protocols for this dissertation project were approved by the human subject research ethics committees for UNC, the Galápagos Science Center and Universidad de San Francisco de Quito.
5.1. Fieldwork

Two rounds of recruitment were conducted during field seasons one and two. A total of 169 children ages two to ten years old and 119 of mothers or caregivers (3 grandmothers) were sampled from 12 neighborhoods in Puerto Baquerizo Moreno, the capital of Galápagos, which constitutes the densely populated urban zone of San Cristóbal. This represents 14% of the town’s population of children under ten years (CGREG 2013). We recruited mothers by going door-to-door in each neighborhood with the highest number of children (according to local informants) until saturation was reached. We anticipated and met a high rate of participation based on mothers’ expressed interest in public health research (Page et al. 2013), their concern with a lack of health services (Walsh et al. 2010) and the project’s association with the Galápagos Science Center. Recruitment incentives for mothers included information on the height, weight, body composition and iron status of themselves and their child (and other family members if requested). This provided feedback on health and nutritional status that would not otherwise be available without consultation by a physician. In addition, mothers were informed of their household drinking water quality, which was of high local interest according the results from the pilot study. If fecal pathogen levels were deemed unhealthy according to the World Health Organization’s (WHO) recommendations (WHO 2011b), culturally appropriate preventive strategies were discussed. A total of 71 children and 50 mothers participated in the follow-up study collectively during field season two and three.

Data collection included interviews with mothers about their children and households, anthropometric assessments, one fecal sample, two rounds of blood spots collected approximately ten days apart, and one household tap water sample for each child. After recruitment into the project and informed consent was obtained for each mother and child, they
were visited three times over approximately two weeks to conduct interviews and obtain necessary samples. Interviews and sample collection took place in their household unless otherwise requested by the mother.

5.2. Visit One

During the first visit, mothers were interviewed and given detailed instructions for the child’s fecal sample collection. We performed anthropometric assessments and collected the first round of blood spots from the child and water samples for each household.

Mother’s Interview: Each mother or caregiver was asked a structured oral questionnaire in Spanish and with relevant sections on 1) household socio-demographics, 2) household water and sanitation characteristics, 3) child’s demographics including birth, breastfeeding, weaning and health information and 4) child’s hygiene practices including bathing, hand washing and defecation, 5) one-week symptom history and 6) 24-hour diet food recall and food frequency questionnaire. The one-week child symptom history section asked whether the child experienced any symptoms of diarrhea, cough, vomiting, urinary tract infection, skin rash, allergies and other possible illnesses within the past week, along with their duration. The dietary recall asked for all types and quantities of foods, drinks and medicines consumed by the child within the past 24 hours (Buzzard 1998).

Water Sample: We collected a small 100mL sample of household tap water in a sterile container, which was tested for the presence of *E. coli* and total coliforms at the Galápagos Science Center. Results of the baseline test were given to the mothers during the second visit. If levels are deemed unhealthy according to the WHO recommendations (WHO 2011b), preventive practices, such as boiling water, washing fresh produce and hand washing, were discussed. Since it is well known that the municipal water supply is contaminated, households rely on purchasing
bottled water for drinking and sometimes cooking uses. During field season one, we also collected household *drinking* water samples and tested for *E. coli* and total coliforms.

**Round One Blood Spots:** Blood samples were collected during visit one and three, after approximately ten days, from each child. A minimally invasive 50μL blood sample was obtained by pricking the finger with a sterile, disposable microlancet (pin prick). This typically resulted in three to five drops of blood. One drop was analyzed for hemoglobin levels in the field using the HemoCue HB201 point-of-care device (HemoCue Cypress, CA), which indicated iron status. The hemoglobin results were immediately given to the mothers. If iron status was low we referred them to the free public hospital. The rest of the drops were stored and allowed to dry on protein saver cards (Whatman 903) (McDade et al. 2007). Once dried, the cards were sealed in plastic bags with packets to absorb moisture and stored in the freezers at the Galápagos Science Center until assayed.

**Fecal Sample:** Each mother was given oral and written detailed instructions for the collection of the fecal sample, and a sample collection kit including gloves, a paper plate, a spatula, a small container similar to ones given at the doctor’s office and a sealed ziptop bag to store the sample. Mothers were asked to collect a small sample of feces (400mg, roughly the size of a vitamin) and store it in the provided containers inside their freezers. If freezers were not available or they did not feel comfortable, we provided them with insulted bags and ice-packs. We checked with the households each day following visit one to pick up the sample. Once we collected the fecal samples, they were then stored in freezers at the Galápagos Science Center until transported to UNC for analysis after the completion of fieldwork.

**Urine Sample:** Based on the high rates of reported urine tract infections during field season one, we decided to collect urine samples during field season two for urinalysis. Each
mother was given oral and written instructions for the collection of urine for both themselves and their child, including a sample collection kit with gloves, hygiene wipes, sealed bags and urine collection containers similar to those given at the doctor’s office. They were asked to collect the required amount specified on the container the following morning. The sealed container was then stored in their refrigerator or provided insulated cooler until it was picked up later that day.

**Anthropometric Assessment:** Height, weight, waist and mid-upper arm circumference and triceps, subscapular and suprailiac skinfolds were taken from each child following standardized methods (WHO 1995). Only height and weight were taken from mothers. Portable equipment was used including a SECA stadiometer, an electronic scale, measuring tape and Lange skinfold calipers. These results were given to the mothers or caregivers immediately.

**Blood Pressure Assessment:** Blood pressure assessments were taken from mothers using an automated Omron wrist monitor.

5.3. **Visit Two**

The following day, we went by each household interviewed the previous day to check whether they had collected the fecal and urine samples. If ready, we collected them immediately and if not, we continued to check with them daily to arrange for their pickup.

5.4. **Visit Three**

After approximately ten days from the visit one, we returned to each household to collect the second round of blood spots. Mothers were again given the one-week symptom questionnaire to account for illness between blood spot collections. The results from the household water quality test were given to the mothers. To assess variation in water quality over time, we collected and analyzed an additional household tap water sample during field season two.
5.5. **Follow-Up Study**

During the following field season after recruitment and baseline interviews (including visits one to three), we attempted to follow-up with each household to collect a second round of anthropometrics to determine linear growth and weight gains, and collect additional blood spots. Growth and weight gain results were given to the mothers immediately. One final household tap water sample was collected and analyzed to assess for long-term changes in water quality. Follow-up data was collected for a total of 71 children and 50 mothers.

5.6. **Key Study Measures**

**Household Water Quality:** *Escherichia coli* levels are a highly reliable of measures of fecal contamination of water sources and are an indicator of inadequate sanitation (Edberg et al. 2000). Household water samples were tested for the presence and most probable number (MPN) of *E. coli* and total coliforms using the culture-based IDEXX Colilert test (IDEXX Laboratories, Inc. Westbrook, Maine) at the Galápagos Science Center (Cochran 1950; Eckner 1998). Reagents were added to each sample and sealed in the IDEXX counting tray. After incubation, the sample was fluoresced to obtain the upper and lower bacterial concentrations using the Quanti-tray 2000’s most probable number methodology (IDEXX Laboratories, Inc. Westbrook, MA). Based on the WHO’s recommendations, household water samples were categorized based on potential health risk (Havelaar et al. 2001; WHO 2011b) and were used as the primary pathogenic predictor variable in Pilot Aim 2 and Dissertation Papers 2 and 3.

**Blood Immune Biomarkers:** Three rounds of blood spots were assayed for CRP and EndoCAb levels using double sandwich enzyme-linked immunosorbent assay (ELISA) technique at the Galápagos Science Center’s microbiology laboratory. Eluted blood spots were analyzed using Quantikine’s Human High Sensitivity C-Reactive Protein ELISA (R&D Systems,
Inc. Minneapolis, MN) (McDade et al. 2004) and Hycult’s EndoCAb IgG ELISA kit (Hycult Biotech Inc. Plymouth, PA) (Barclay 1995). CRP cut-points for low (<1 mg/L), moderate (1 to 3 mg/L) and elevated inflammation (3 to 10 mg/L) are taken from clinical practice (Pearson et al. 2003; Ridker 2003). Children with acute levels (>10 mg/L) are considered to have infections. Since there are no standardized cut-points for endotoxemia using EndoCAb IgG levels, the 75th percentile expressed as median unit (MU) per mL was used as the reference point for high microbial translocation (Barclay 1995). These blood immune function measures were examined as indicators of immunodysregulation for both the Pilot and Dissertation Aims, along with the primary outcomes in all three Dissertation Papers.

**Iron Status:** Hemoglobin status indicating iron deficiency was determined using the WHO age-specific cut-points for children women (WHO 2011a). Iron deficiency was used as a dietary predictor of nutritional deficiency for Pilot Aim 2.

**Gut Microbial Composition:** DNA was isolated using protocols from the Qiagen BioRobot Universal (Qiagen, Valencia, CA) and was quantified using Quant-iT™ PicoGreen® dsRNA Reagent (Molecular Probes, Life Technologies division of Thermo Fisher Scientific, Waltham, MA). A Roche GS FLX Titanium instrument was used to perform 16S rDNA bacterial amplicon pyrosequencing (Microbiome Core Facility, UNC). The QIIME pipeline (Caporaso et al. 2010) was used to analyze sequencing data and assign operational taxonomic units (OTUs). Gut health was assessed by exploring each taxonomic level to identify relevant microbial compositions related to pathogenic and obesogenic risk factors, as well as influence on immune function in both Pilot Aims and Dissertation Papers. We chose to analyze microbial compositions at the family level because it allows for a broad array of bacterial types without
being too extensive, and is the primary outcome used in Paper 3- Gut Microbiota Mediate Immunostimulation.

**Urine Immune Biomarker:** Urinalysis was performed on the urine samples at the Galápagos Science Center using URS-11 reagent strips (Cortez Diagnostics, Inc., Calabasas, CA) to determine levels of leukocytes, nitrates and blood indicative of urinary tract infections. These urinary immune function measures were used to complement blood immune function measures for outcomes and to adjust for infection in the Pilot and Dissertation Aims.

**Adiposity and Growth:** Body mass index (BMI) was calculated using mass(kg)/height(m)². Sex- and age- specific z-scores were calculated using the WHO reference data and cut-points for comparison across sex and age (de Onis and Lobstein 2010; de Onis et al. 2006; WHO 2006). Height-for-age z-scores were used to assess linear growth restrictions. BMI-for-age, skinfold and waist circumference z-scores were used to assess adiposity. These variables were examined as the main adiposity and linear growth outcomes for Pilot Aims and Dissertation Aim 2. Obesity was defined by a BMI z-score over 2 and used to adjust models in Papers 2 and 3, and used as the sensitivity and specificity predictor in Paper 1 - Measuring Chronic Low-Grade Inflammation.

**Blood Pressure:** Diastolic and systolic blood pressure was determined for each mother and risk of hypertension was evaluated using American Heart Association’s cut-points (AHA 2017). These measures were explored to determine chronic disease risk associated with obesogenic factors among women in the Pilot Aims.

**Household Sanitation:** Using data from mothers’ interviews, household sanitation measures were created, including housing type, drinking water source, household water use,
water treatment, bathroom type, trash disposal and the presence of domesticated animals near the household. These variables were explored and used as pathogenic predictors in Pilot Aim 2.

**Household Socio-Demographics:** Data from mothers’ interviews were used to create household socio-demographics variables, including parental marital status, family size, mother’s education, parental employment, home ownership, land ownership elsewhere on the island, household assets, co-residence of grandparents, and smoking in the household. These measures were used as social predictors in Pilot Aim 2.

**Child’s Demographics:** Age, sex, ethnicity, birth weight, delivery mode, birth place, breastfeeding practices, formula use, introduction to solid foods, and school attendance were created from mothers’ interviews and used as the demographic predictors in Pilot Aim 2. Age and sex variables were used to adjust all aims and papers.

**Child’s Hygiene:** Data from mothers’ interviews were used to create hygiene variables, including the toilet use, diaper use, bathing water source, oral hygiene practices, hand washing practices, frequency of swimming in the ocean, last deworming and interactions with animals. These variables were used as pathogenic predictors in Pilot Aim 2.

**Infectious Symptoms:** Infectious symptom measures were created using data from the two one-week symptom histories taken during visits one and three. The presence of diarrhea, fever, vomiting, cough, cold, urinary tract infection, skin rash, allergies and asthma were used as pathogenic predictors in Pilot Aim 2. If mothers reported that a child experienced diarrhea, vomiting and fever during either visits one or three, the child was considered to have infectious symptoms used to adjust and stratify models in Papers 2 and 3.

**Dietary Composition:** Data from food frequency questionnaires administered during field season two were used to calculate reported average consumption of high-fiber foods, sugary
drinks, dairy products, fried foods and other local unhealthy food items. Consumption was categorized into high and low quantities and used as the main dietary predictor for Pilot Aim 2. Alternative to dietary intake, food preferences for field season two and household staple food items for the entire sample were explored as dietary predictors.
CHAPTER 6. PAPER 1- MEASURING CHRONIC LOW GRADE INFLAMMATION

_Can cross-sectional measures of C-reactive protein adequately estimate chronic, low-grade inflammation in populations living in dual burden environments?

6.1. Introduction

C-reactive protein (CRP) is an acute phase protein of the inflammatory immune response with the primary purpose of responding to infection or injury. Short-term elevations in CRP over 10mg/L indicate clinical infections and can occur within hours and remain elevated until the infection is cleared or treated (Grønn et al. 1986). The production of CRP is stimulated by the pro-inflammatory cytokine interleukin-6 (Il-6) and can rise to 5mg/L six hours after pathogenic exposure, peaking at 48 hours (Pepys and Hirschfield 2003). CRP has a half-life of 19 hours and is cleared at a constant rate regardless of infection type (Vigushin et al. 1993). Thus the acute phase response to non-specific infection results in high variability of CRP levels within an individual during the active infection and resolution period.

Moderately elevated, stable levels of high-sensitivity CRP from 3-10mg/L, on the other hand, indicate chronic, low-grade inflammation and are highly correlated with increased risk of cardiovascular disease and stroke among adults (Ridker 2007; Rifai and Ridker 2001). Even among children, moderate elevations of CRP are related to indicators of cardiovascular health such as HDL cholesterol and systolic blood pressure (Cook et al. 2000). In settings with low infectious disease burdens, individual CRP levels are considered stable over time (Macy et al. 1997; Ockene et al. 2001; Platz et al. 2010) and the principle driver of moderate elevations is adiposity (Park et al. 2005), not infection. Consequently, intra-individual variability due to infection is rarely examined or measured in epidemiology studies of chronic inflammation,
obesity and cardiovascular disease risk in high income countries. In 2003, the Centers for Disease Control and Prevention (CDC) and the American Heart Association (AHA) recommended that in order to use CRP as an indicator of cardiovascular disease risk in the clinical setting two measures should be taken approximately two weeks apart and averaged, with retesting of levels over 10mg/L (Pearson et al. 2003). Despite these recommendations, the common practice in population studies is to use a single measure of CRP and exclude individuals over the 10mg/L acute cut-point (e.g. National Health and Nutrition Examination Survey (Ford et al. 2003)), possibly due to logistical and financial limitations. The biologic variability of CRP, especially in relation to infection, is a crucial factor influencing the validity of its use as an indicator of chronic, low-grade inflammation and needs further investigation (Braga and Panteghini 2012).

The purpose of this paper is to assess whether the standard use of a single CRP measure is sufficient, or if multiple measures used to account for intra-individual variability are needed to adequately capture chronic low-grade inflammation among populations living in high pathogenic environments coupled with overnutrition, conditions that are increasingly common in low and middle income countries. Using the clinical cut-point of CRP values over 10mg/L from a single measure to indicate individuals experiencing infection commonly used in high income countries could be problematic in populations with greater infectious disease burdens (McDade et al. 2012a). With higher infection rates, individuals are more likely to exhibit elevated CRP levels with a single measure taken during active infection (>10mg/L) or the inflammatory resolution phase (<10mg/L). This methodology may overestimate chronic, low-grade inflammation and risk of cardiovascular disease at the population level. In these environments, longitudinal measures may be needed to determine intra-individual variability in response to infection.
Using sensitivity and specificity analyses in relation to obesity, this study examines the accuracy of four different methods for excluding individuals with probable infections when estimating moderately elevated inflammation (Table 6.1). Levels of inflammation are measured using two CRP measurements taken at baseline (time-1) and again after approximately ten days (time-2) in 113 women and 159 children from Galápagos, Ecuador. The first method examined is common in epidemiology and uses the cross-sectional measures at time-1 and time-2, separately, excluding individuals over the clinical cut-point of 10mg/L for acute infection. The next method is also taken from population studies and uses a single measure of CRP at time-1 and discards observations with self-reported infectious symptoms. The third method is longitudinal and calculates the mean of both time points, removing values over 10mg/L as suggested by the CDC and AHA for clinical use. The last method is unique to this study and also utilizes the mean of both time points, but excludes individuals with a change in CRP of more than 3mg/L between time points. The receiver operator characteristic (ROC) curves and area under the curves are compared for each method to determine their accuracy in predicting obesity as an indicator of cardiovascular disease risk. The clinical range of 3-10mg/L is widely used for indicating moderate inflammation associated with high risk and the more relaxed range of 1-10mg/L is used to include intermediate risk (Ridker 2007). This study compares the sensitivity, specificity and percent correctly classified for all methods using these two ranges to identify which combination of method and range is more precise in excluding elevations related to infection and estimating chronic, low-grade inflammation in the sample. Since adults and children have distinct patterns of risk for obesity and infectious disease (DeBoer et al. 2012), the four methods will be evaluated separately for women and children.
Contrary to the CDC and AHA recommendation of using the mean of two measures excluding values over 10mg/L (Pearson et al. 2003) or other common practices using single measures, we hypothesize that the mean CRP value of the two time points excluding high change over 3mg/L will be more precise in identifying and removing elevated CRP levels due to infections. We expect that excluding individuals with high CRP change will better capture those undergoing an acute phase response to infection, compared to excluding those over the 10mg/L cut-point. Individuals recovering from an acute infection in the resolution phase may still have elevated levels under the 10mg/L cut-point. This study proposes that excluding high change will provide a more accurate measure of moderately elevated CRP, inferring the prevalence of chronic, low-grade inflammation in a dual burden environment. Similar to developing countries, residents of Galápagos are undergoing rapid nutrition transition resulting in increased rates of obesity (Freire et al. 2014a), alongside high infectious disease burdens caused by poor water quality and inadequate sanitation infrastructures (Gerhard et al. 2016; Page et al. 2013; Walsh et al. 2010). This study is the first to estimate chronic, low-grade inflammation among women and children in Galápagos, Ecuador.

6.2. Sample and Data Collection

Data are drawn from an anthropological study investigating the impact of early life nutritional and disease environments on immune function and intestinal health among residents of the island of San Cristóbal. One hundred and thirteen women aged 17 to 57 and their 159 children aged two to ten were sampled. Mothers were recruited door-to-door from 12 neighborhoods in the urban area of Puerto Baquerizo Moreno until neighborhood saturation was met. Interviews were conducted during two field seasons between June 2013 and May 2014 to obtain demographic information, perform anthropometric assessments and collect blood samples.
This research received human subject ethics approval by the University of North Carolina, Chapel Hill (UNC) and Universidad de San Francisco de Quito, Ecuador.

6.3. Measures

**Obesity:** Women and children were weighed and their standing height was measured following standard protocols (WHO 1995) using a portable scale and stadiometer during the baseline visit. Body mass index (BMI) was calculated using weight(kg)/height(m)². The World Health Organization’s (WHO) growth references were used to calculate BMI-for-age z-scores for children (de Onis et al. 2007; WHO 2006). Obesity was defined as a BMI-for-age z-scores of two or above for children and a BMI of 30 or greater for women.

**CRP Assays:** Two blood samples were taken from each participant. One at baseline and a second after approximately ten days. A minimally-invasive finger-stick was used to draw blood spots, which were collected on protein-saver card (Whatman 903), allowed to dry and then stored in freezers for subsequent laboratory analysis. Dried blood spot methods for CRP have been previously validated and found to be relatively reliable and accurate (McDade et al. 2007). Each sample was measured for high-sensitivity C-reactive protein (CRP) levels, as an indicator of inflammation, using a double sandwich enzyme-linked immunosorbent assay (Quantikine CRP immunoassay, R&D Systems, Inc. Minneapolis, MN). The detectable range of CRP was .003 to 13.030mg/L, with an inter-assay coefficient of variation of 10%. Assays were performed in the microbiology laboratory at the Galápagos Science Center, San Cristóbal, Ecuador.

**Measures of Chronic, Low-Grade Inflammation:** Four methods for detecting chronic, low-grade inflammation using cross-sectional and longitudinal CRP values were identified *a priori* (Table 6.1). Measures 1 and 2 use the first method of removing values over 10mg/L analyzed from cross-sectional collection at time-1 and time-2, respectively. Measure 3 also uses
the cross-sectional levels of CRP at time-1 and discards observations where participants reported having at least one infectious symptoms of diarrhea, fever or vomiting within the last week. Measure 4 is longitudinal and uses the mean values of both time points, excluding single values over 10mg/L and replacing the mean with the remaining value. Finally, measure 5 uses the mean of two time points excluding individuals when the difference between the time points is greater than or equal to 3mg/L. Using the previously defined ranges of 3-10mg/L for high risk of cardiovascular disease and also a more relaxed range of 1-10mg/L to include intermediate risk (Pearson et al. 2003; Ridker 2007), the prevalence of moderately elevated CRP is calculated using each measure for women and children, separately.

6.4. Statistical Analyses

To evaluate the accuracy of each of the five measures of moderately elevated CRP for distinguishing between normal and obese individuals based on the four different methods of excluding individuals with potential infection, we analyze the receiver operator characteristic (ROC) curves for women and children. Sensitivity and specificity analyses using ROC curves can evaluate the diagnostic abilities of an indicator variable to predict an outcome (Florkowski 2008). Sensitivity is calculated as true positives/(true positives + false negatives) of the indicator at a given cut-point in predicting the outcome, and represents the rate of true positives. For example, the sensitivity in this analysis provides the probability that a given cut-point of CRP will correctly identify obese individuals, when the individuals are in fact obese. Specificity represents the rate of true negatives and is calculated as true negatives/(true negatives + false positives). In this study, specificity provides the probability that CRP at a given cut-point will predict that an individual is not obese, when they actually are not obese. ROC curves graphically display the relationship between sensitivity and 1-specificity (rate of false positives) of an
indicator variable’s ability to predict an outcome for all possible cut-point values of that indicator. The area under the curve provides a measure of the indicator’s ability to discriminate between the presence and absence of the outcome and represents the average true positive rate for the distribution of all cut-points.

First, non-parametric estimates of the ROC curves were examined for each measure (Table 6.1) in women and children, regardless of cut-points. To test the hypothesis that the measure 5, mean excluding high change, is a more precise estimation of moderately elevated CRP excluding elevated levels of infection, the area under the ROC curve was calculated and we compared each measures’ ability to discriminate between obese and normal individuals. The estimated areas under the curve were determined to be significant at a 95% confidence level and differences were evaluated using test of equality for different subsamples (Cleves 2002). Next, each measure of moderately elevated CRP was evaluated for sensitivity, specificity, and the percent correctly identified using both the 3-10mg/L and 1-10mg/L ranges. Measures in relation to specific ranges were thought to be sufficient in estimating chronic, low-grade inflammation if they exhibited 50% sensitivity and 70% specificity, with over 60% correctly classified. A higher level of specificity was chosen to lessen the chance of incorrectly identifying ambiguous CRP levels as elevated in relation to obesity.

6.5. Results

The mean age of women was 30 years and approximately 31% were obese. Among children aged two to ten, 21% of the 81 boys and 15% of the 78 girls were obese. Figures 6.1 and 6.2 display the mean and intra-individual ranges of CRP levels between the two time points for women and children, respectively. Each dot represents the mean value between time points, and are arranged by rank order of mean CRP level. Each bar displays the range of variation for
individuals between time points. Grey bars indicate that the individual experienced a change in CRP levels over 3mg/L, suggested by measure 5. There was considerable variation in means and intra-individual variability in both children and women. Dots falling below the 1mg/L or 3mg/L cut-points with short bars indicate stable, low CRP levels suggesting no increased risk of cardiovascular disease associated with chronic inflammation.

Approximately one quarter of the 159 children demonstrated high variability between the time points (see the right side of the distribution in Figure 6.2), indicating an acute response to infection or injury. Although there was more inter-individual variability between women, there were a considerable number of women who experienced a high range of variation between time points. Among both women and children, some individuals who experienced high variability, indicating elevations due to infection, had both CRP levels fall below the 10mg/L clinical cut point. If using a cross-sectional method described in measures 1 and 2, or the mean measure 4 excluding level over 10mg/L, these individuals would be considered to have moderately elevated CRP levels with high risk for cardiovascular disease.

The areas under the ROC curve are listed for all measures among women and children in Table 6.2. Measure 5, mean excluding high change, had these highest ROC areas for both women and children, indicating that this measure provided the greatest level of discrimination between normal and obese individuals compared to other measures. The average rate of true positives for this measure was 70% among women and 80% among children. The cross-sectional measures 1 and 2 discarding CRP levels over 10mg/L provided the second largest areas under the ROC curve for women at 66% and children at 77%. Yet in children, measure 1 had the lowest discriminating ability, and in women, measure 2 tied for the lowest area under the curve.
In comparing areas under the ROC curve for each measure, we found no statistical differences between measures for women or children.

Tables 6.3 and 6.4 report the sensitivity, specificity and percent correctly classified of each measure using the 3-10mg/L and 1-10mg/L ranges in women and children, respectively. Among women, no measure using either range met our criteria of 50% sensitivity, 70% specificity and 60% correctly classified. Although the specificity and percent correctly classified for the 3-10mg/L range was sufficient for measure 2, cross-sectional at time-2, and measure 5, mean excluding high change, sensitivity was low at 28% and 18%, respectively. For the 1-10mg/L range for all measures in women, the sensitivity was higher, which lowered specificity. For measure 5, mean excluding high change, the sensitivity and percent correctly classified met the criteria, yet the specificity was only 52%.

Among children, the measures using the range of 3-10mg/L to indicate moderately elevated CRP exhibited unbalanced sensitivity and specificity. Sensitivity levels were all under 14% while specificity levels were over 91%, and the percent correctly classified were also high. The range of 1-10mg/L yielded greater balance between sensitivity and specificity. Both longitudinal measures met our criteria for estimating moderately elevated CRP. Measure 4, mean excluding levels over 10mg/L, demonstrated 55% sensitivity, 80% specificity and 74% correctly classified, whereas measure 5, mean excluding high change, exhibited 52% sensitivity, 88% specificity and 81% correctly classified.

According to measure 5, mean excluding high change, with the range of 1-10mg/L, the estimated prevalence of moderately elevated CRP in children from Galápagos was 20% compared to 24% using measure 2, cross-sectional CRP at time-2. Although no measures among women satisfied our selection criteria for adequate sensitivity-specificity balance, measure 2
using the 3-10mg/L range provided the closest specifications. Using this measure and range, the estimated prevalence of moderately elevated CRP was 24%.

6.6. Discussion

Women and children living in Galápagos demonstrated high levels of inter- and intra-individual variability in CRP. Such intra-individual variability over ten days, especially among children, is likely caused by an acute phase response to infection or injury. This pattern was expected given the high pathogenic environment on San Cristóbal associated with poor water and inadequate sanitation infrastructures (Gerhard et al. 2016), and has also been demonstrated in an adult indigenous population in the Ecuadorian Amazon (McDade et al. 2012a). This pattern of variability is in contrast to studies in the US and other high income countries, where CRP levels are typically considered to be stable over time and where intra-individual variation is minimal (Chen 2009; Macy et al. 1997; Ockene et al. 2001; Parrinello et al. 2015). Yet, unlike the indigenous Amazonian population and more similar to the US population, approximately 24% of women and 20-24% children from the Galápagos demonstrated some evidence of chronic, low-grade inflammation based on sensitivity-specificity analyses of measures and ranges used in this study.

We found that the longitudinal method of excluding observations of high change resulted in the greatest area under the curve for both women and children compared to other methods. These findings support our hypothesis that this measure of excluding high CRP change was more precise than the other measures at identifying and discarding elevations due to infection when estimating moderately elevated CRP. However, we found no statistical differences between the estimated area under the curve for any measures in women or children. These results suggest that, while the method of using the mean excluding high change provides more discriminating
abilities in regards to obesity, the other measurements may be sufficient at estimating chronic, low-grade inflammation at a given cut-point. Among cross-sectional measures, time-2 had the greatest area under the curve in women, whereas time-1 had the greatest area under the curve in children. This may suggest that while not statistically different, using a single measure of CRP at baseline to estimate moderately elevated inflammation may not be the most reliable.

The irregularity of using cross-sectional measures is demonstrated by our findings that a considerable proportion of women exhibited levels above and below the 3mg/L cut point between time points (Figures 6.1 and 6.2). Evidence from the multi-ethnic study of atherosclerosis (MESA) also demonstrated significant intra-individual variability in adult CRP levels, as compared to measures of total cholesterol and non-HDL cholesterol (DeGoma et al. 2012). While excluding individuals with levels over 10mg/L or a history of infection, asthma and other inflammatory disorders, 38% of the variance in CRP was attributed to intra-individual factors between three time points at baseline, 24 and 48 months. More concerning is the finding that 69% of adults in the high-risk 3-10mg/L range at baseline fell to below 3mg/L at follow-up time points, signifying that a substantial proportion considered high-risk at baseline might have had moderately elevated levels caused by infection or injury, unrelated to obesity or risk of cardiovascular disease.

In another study of CRP variability over a four week period, high variability related to high infectious disease burdens was found among the indigenous Shuar living in the Ecuadorian Amazon (McDade et al. 2012a). Although the study did not exclude individuals over 10mg/L, approximately 95% of the variance in the four CRP measures was due to characteristics within the individual that change over time, such as having an infection. While 35% of individuals who had CRP levels in the 3-10mg/L range at one measure, no individual demonstrated a repeated
measure in the high risk range, indicating that those individuals were not experiencing chronic, low-grade inflammation as would have been reported using cross-sectional measures. Using single measures to assess moderately elevated CRP, even in low infectious disease burden countries, may be capturing individuals undergoing an acute phase response due to infection and over estimating the prevalence of chronic, low-grade inflammation.

We found that no measure in combination with either the clinical 3-10mg/L or the more relax 1-10mg/L ranges met our sensitivity and specificity criteria among women. Using the clinical range, levels of sensitivity were too low, and conversely using the relaxed range led to low levels of specificity. When sensitivity is low, the measure is weak at correctly identifying individuals without the condition, and when specificity is low, the measure is weak at correctly identifying individuals with the condition. To provide a more conservative estimate given the condition of high rates of infections, we chose to limit false positives and allow for lower sensitivity using the clinical range. The cross-sectional measure at time-2 provided the highest level of sensitivity while meeting our specifications for specificity and the percent correctly classified. Research in China has shown that both pathogenic and obesogenic factors independently increased relative risk of moderately elevated CRP and acute CRP separately in adults (Thompson et al. 2013). This suggests that accurate estimation of the prevalence of chronic, low-grade inflammation using cross-sectional measures among populations living in conditions of the dual burden environment, with high infectious disease burdens and overnutrition, presents even more challenges in attempting to increase specificity.

Similarly among the indigenous Tsimané population in Bolivia, 50% of adults over 40 years fell within the 3-10mg/L range, suggesting a high percentage of individuals were at risk of cardiovascular disease (Gurven et al. 2009). However, only 4% adults had hypertension
indicating the measure had a high rate of false positives. Furthermore, 23% were over the 10mg/L CRP cut-point for clinical infection, 17% had elevated white blood cell counts and 82% had elevated erythrocyte sedimentation. This demonstrates that high levels of immune activation due to infection, and not chronic, low-grade inflammation driven by obesity caused the elevated CRP levels. While less evident in rural Ghana, 12% of older females and 19% of older males had moderately elevated CRP; however, with a 78% malaria infection rate among the population, it is likely that these elevations are not associated with cardiovascular disease risk (Koopman et al. 2012). Taken with the results of this research, these studies demonstrate the necessity of using repeated measures of CRP to assess for acute changes in highly pathogenic environments to more accurately identify risk of chronic, low-grade inflammation and prevent overestimations.

Among children, both longitudinal measures using the mean between time points and discarding values over 10mg/L or values with a high change of 3mg/L were found to be sufficient measures using 1-10mg/L range. This may be due to the fact that the distribution of high intra-individual variability among children was more consistent, with fewer children experiencing intermediate levels of variability and overall less moderately elevated CRP levels associated with obesity. This reduced the probability of misclassifying ambiguously elevated CRP levels as being related to obesity, thus allowing for higher levels of specificity. Although both longitudinal measures fit our sensitivity and specificity criteria, the method using the mean between time points and discarding high change demonstrated a 7% higher level of individuals correctly classified, suggesting that it provides a more precise estimate.

Using single measures to assess for moderately elevated CRP indicating chronic, low-grade inflammation in epidemiology studies may be adequate in populations with previously demonstrated low intra-individual variability. However in populations experiencing a high
infectious disease burden typical of low and middle income countries, it is methodologically challenging because of high levels intra-individual variability due to pathogenic exposure. Even when using longitudinal measures of CRP in this study, some women and children experiencing an acute reaction to infections identified by high variability did not have at least one measure over the 10mg/L clinical cut-point. According to the CDC and AHA recommendation of calculating the mean of repeated measures excluding acute levels over 10mg/L, both measures would have been used, leading to an overestimated mean value. If that mean value was within the 3-10mg/L range, as it was for some women in this study, it would result in a higher prevalence of chronic, low-grade inflammation.

In recognition of this issue of overestimation among populations with high disease burdens, we suggest that using the mean values of repeated measures excluding high change of over 3mg/L may better capture and exclude individuals experiencing acute infectious reactions. We hypothesized that this measure would have the greatest area under the ROC curve compared to all other measures since it is more robust in excluding individuals with possible acute infections. Thus, the individuals captured in the category may more accurately reflect those with obesity-associated elevations. Surprisingly, we did not find any statistically significant differences between the areas under the ROC curves calculated for each measure. This suggest that although cross-sectional measure may not provide as reliable estimates as longitudinal measures, they may be sufficient given a specific cut-point. However among women in Galápagos, the use of a single measure while using the 3-10mg/L clinical range for moderately elevated CRP was not sufficient in predicting chronic, low-grade inflammation associated with cardiovascular disease risk.
This study was limited by a number of factors. First, we assess the accuracy of each measure in its ability to discriminate between normal and obese individuals. This assumes that all chronic, low-grade inflammation indicating elevated cardiovascular risk is exclusively associated with elevations due to obesity. Although excess adiposity provides a site for increased production of the pro-inflammatory cytokine IL-6 (Després 2012), chronic elevations of CRP due to low-grade infections may also contribute to the inflammatory etiology of heart disease. This study would be strengthened by testing the diagnostic capabilities of our measures in predicting other indicators of cardiovascular disease risk, such as hypertension and cholesterol levels (Hage 2014; Ockene et al. 2001). In addition to examining intra-individual variability to identify CRP elevations caused by infections, the examination of other pro-inflammatory cytokines and immune function measures would have increased our ability to detect individuals undergoing immunostimulation due to pathogenic exposures. Similarly, our study was limited to two repeated measures of CRP over approximately ten days, which may not have fully captured all elevations due to infection considering the half-life of CRP. Lastly, it is unclear whether our findings can be generalized to other dual burden populations or other areas of Ecuador. More research is needed to assess the functional ability of using cross-sectional and repeated measures of CRP to estimate chronic, low-grade inflammation in high infectious disease burden populations.

6.7. Conclusion

To our knowledge, this study was the first to assess inter-individual variation in CRP levels among women and children from a population experiencing a dual burden of increasing rates of obesity and cardiovascular disease, coupled with persistently high rates of infections. Our findings indicate a moderate presence of both acutely elevated inflammation due to
infections and chronic, low-grade inflammation in children and women in Galápagos, Ecuador. Further research is needed to incorporate additional inflammatory cytokines and specific indicators of cardiovascular disease risk to test the predictive strengthens of our measures of moderately elevated CRP. However, the significance of these findings caution that the common use of cross-sectional measures and the recommendation by the CDC and AHA for using the mean values and excluding values >10mg/L may lead to overestimated prevalence of chronic, low-grade inflammation in adults and children that are not associated with obesity and cardiovascular disease risk. This is especially relevant among populations with high infectious disease burdens. To alleviate this problem, we suggest using the mean of two CRP values, excluding those with a change over 3mg/L, when estimating the prevalence of moderately elevated CRP levels.
Figure 6.1 Mean and range of variation of CRP between time-1 and time-2 for women.

Figure 6.2 Mean and range of variation of CRP between time-1 and time-2 for children.
Table 6.1 Measures of moderately elevated CRP using four different methods of discarding

<table>
<thead>
<tr>
<th>#</th>
<th>Measures</th>
<th>Definition</th>
</tr>
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<tr>
<td>1</td>
<td>Time-1</td>
<td>Cross-sectional measure at time-1, discarding values &gt;10mg/L</td>
</tr>
<tr>
<td>2</td>
<td>Time-2</td>
<td>Cross-sectional measure at time-2, discarding values &gt;10mg/L</td>
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<tr>
<td>3</td>
<td>No infectious symptoms</td>
<td>Cross-sectional measure at time-1, discarding observations with self-reported infectious symptoms</td>
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<tr>
<td>4</td>
<td>Mean</td>
<td>Longitudinal measure of the mean values, discarding values &gt;10mg/L</td>
</tr>
<tr>
<td>5</td>
<td>Mean excluding high change</td>
<td>Longitudinal measure of mean values, discarding measures experiencing a change of +/- 3mg/L or values &gt;10mg/L</td>
</tr>
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</table>

Table 6.2 Area under the ROC curve for all measures in women and children

<table>
<thead>
<tr>
<th>#</th>
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<th>Women</th>
<th>Children</th>
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<td></td>
<td></td>
<td>n</td>
<td>ROC area</td>
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<tr>
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<td>Time-1</td>
<td>106</td>
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</tr>
<tr>
<td>2</td>
<td>Time-2</td>
<td>109</td>
<td>0.661</td>
</tr>
<tr>
<td>3</td>
<td>No infectious symptoms</td>
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<td>0.649</td>
</tr>
<tr>
<td>4</td>
<td>Mean</td>
<td>112</td>
<td>0.649</td>
</tr>
<tr>
<td>5</td>
<td>Mean excluding high change</td>
<td>90</td>
<td>0.695</td>
</tr>
</tbody>
</table>

n= total observations

n= total observations; ROC= receiver operator characteristic
<table>
<thead>
<tr>
<th>Measure</th>
<th>Sensitivity 1-10mg/L</th>
<th>Specificity 1-10mg/L</th>
<th>Correctly Classified 1-10mg/L</th>
<th>Sensitivity 3-10mg/L</th>
<th>Specificity 3-10mg/L</th>
<th>Correctly Classified 3-10mg/L</th>
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<td>98%</td>
<td>52%</td>
<td>8%</td>
<td>98%</td>
<td>52%</td>
</tr>
<tr>
<td>Time-2</td>
<td>7%</td>
<td>98%</td>
<td>51%</td>
<td>8%</td>
<td>98%</td>
<td>52%</td>
</tr>
<tr>
<td>No infectious symptoms</td>
<td>7%</td>
<td>98%</td>
<td>51%</td>
<td>8%</td>
<td>98%</td>
<td>52%</td>
</tr>
<tr>
<td>Mean</td>
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<td>98%</td>
<td>60%</td>
<td>8%</td>
<td>98%</td>
<td>52%</td>
</tr>
<tr>
<td>Mean excluding high change</td>
<td>7%</td>
<td>98%</td>
<td>51%</td>
<td>8%</td>
<td>98%</td>
<td>52%</td>
</tr>
</tbody>
</table>

Table 6.4 Sensitivity, specificity and percent correctly classified for measures using the ranges 3-10mg/L and 1-10mg/L in children.
CHAPTER 7. PAPER 2 - E. COLI EXPOSURE AND IMMUNE FUNCTION

Protective effects of E. coli exposure on immunostimulation associated with environmental enteric dysfunction in children from Galapágos, Ecuador

7.1. Introduction

A fundamental shift is occurring within epidemiology, replacing diarrheal disease and pathogen-centered explanations of malnutrition and stunting among infants in the developing world. Now, a more complex understanding of the relationship between intestinal immune health and patterns of growth and development is emerging. Early works hypothesized that chronic immunostimulation, rather than episodes of diarrhea or the suppression of nutrient absorption caused by gastrointestinal infection, was responsible for stunting in resource poor environments (Campbell et al. 2003a; Lunn et al. 1993; Solomons et al. 1993). Environmental enteric dysfunction (EED), previously referred to as environmental enteropathy, is characterized by chronic, systemic immune activation caused by intestinal inflammation and increased permeability, allowing for the translocation of gram-negative bacteria across the intestinal barrier into the blood stream initiating endotoxemia (Watanabe and Petri 2016). Typical in low and middle income countries, EED is attributed to repeated fecal pathogen exposures caused by poor, unsanitary environments with limited access to clean water (Humphrey 2009; Korpe and Petri 2012). However to this date, only a few observational studies have investigated risk factors or effects of poor environmental conditions on EED (e.g. George et al. 2015b). We are aware of no empirical studies that have measured the direct effects of fecal pathogen exposure in contaminated water on immune activation related to EED.
While the majority of EED studies are conducted within low-resource settings with high pathogen environments notorious for unsafe drinking water, such as the Gambia (Campbell et al. 2003b), Bangladesh (Mondal et al. 2012), and Nepal (Panter-Brick et al. 2009), the etiology in relation to water quality remains uncertain. EED is often cited as “poorly understood” with no definitive guidelines for identification or non-invasive diagnostic criteria (Korpe and Petri 2012; Kosek et al. 2014). Several studies from Bangladesh have linked EED with observational measures of household environmental conditions (Lin et al. 2013), contact with animals and visibly dirty hands of caregivers (George et al. 2015b), and geophagy (George et al. 2015a). Despite these findings, a hand-washing intervention targeted at mothers in Nepal failed to improve infant biomarker levels related to EED (Langford et al. 2011). Significant money and efforts have been invested into several large scale EED studies to determine its consequences on growth, vaccine response and cognitions (Acosta et al. 2014), and the impact of improvements in sanitation and hygiene practices (Humphrey et al. 2015). However, the hypothesis that EED is caused by habitual fecal pathogen exposures in water and food during childhood remains untested (Korpe and Petri 2012; Watanabe and Petri 2016).

Our study is the first to systematically measure the effects of exposure to fecal pathogens, estimated by *Escherichia coli* concentrations in household tap water, on childhood immunostimulation associated with EED. Epidemiology theory suggests a positive association between high levels of *E. coli* exposure and increased risk of diarrhea and EED; we hypothesize the relationship is more complex. *E. coli* has a great amount of genetic diversity, and evidence from human studies reveals that both commensal and pathogenic strains can provide protection from other pathogenic overgrowth in the gut (Henker et al. 2007; Lodinová-Žádniková and Sonnenborn 1997; Valentiner-Branth et al. 2003). Early life microbial and pathogenic exposures
provide the necessary environmental inputs for proper immune development (MacGillivray and Kollmann 2014). Further, the evolutionary-based “old friends” hypothesis proposes that chronic exposure to mild or immunoregulatory pathogens help guide development and regulation of the immune system during infancy and childhood (Rook et al. 2014). We test the hypothesis that habitual, low-grade *E. coli* exposure from non-drinking water sources that does not cause diarrhea or infection, provides protection against immune activation of biomarkers for EED among children from Galapágos, Ecuador. Since the immune systems of children with gastrointestinal infections will already be active (Borgnolo et al. 1996), we propose that children with infections and lower levels of *E. coli* exposure will have the highest levels of immunostimulation.

The Galapágos islands are an important research setting to investigate EED in relationship to water quality since access to clean water is a major public health concern (Page et al. 2013; Walsh et al. 2010). Residents rely on drinking bottled or treated water because of high levels of fecal contamination of the municipal water supply. A water quality study on San Cristóbal found that in 2013 90% of point-of-use sites were classified as having high or very high health risk from microbial contamination (Gerhard et al. 2016). In 2010, 90% of the island households received this piped water from the municipality, yet an additional 44% purchased 20-liter bottles from two private companies on the island, 39% boiled their water, 8% treated it with chlorine, and 5% had their own filters for drinking use (CGREG and INEC 2010). Although residents have access to cleaner drinking water, they depend on the contaminated municipal water for daily hygiene behaviors and household sanitation practices (Guyot-Tephany et al. 2013). *E. coli* exposures are still likely to occur with contact of fecal-contaminated water used for washing hands, brushing teeth, bathing, washing dishes and consuming raw fruits and
vegetables rinsed with unclean water. Consequently, the island’s public hospital reports high rates of water-borne illnesses and other gastrointestinal infections, with a 12% increase between 2006 to 2009 (CGREG 2010a). Among children under five years, 8% experienced acute diarrhea and 53% experienced respiratory infections within two weeks of a health and living conditions survey in 2010 (CGREG and INEC 2010).

7.2. Sample and Data Collection

Data for this analysis are from a study investigating childhood intestinal health and immune function of mothers and children living on the island of San Cristóbal, Galapágos. Baseline data collection took place over two periods in June-August 2013 and January-May 2014. Mothers of children aged two to ten years were recruited door-to-door in 12 barrios of the Galapágos capital town of Puerto Baquerizo Moreno, until saturation was met in each neighborhood. This analysis uses data from 118 mothers, along with their 166 children. Mothers were interviewed and detailed information was collected about their children’s birth, diet, illness history, and hygiene behaviors, along with household demographics, socioeconomic status, sanitation practices, and water use and availability. For each child participant, anthropometric assessments were performed and two blood spot samples were collected to measure biomarkers of inflammation and intestinal barrier function. Household tap water samples were collected at baseline to determine levels of E. coli contamination. All blood and water analyses were conducted in the microbiology laboratory at the Galapágos Science Center on San Cristóbal, co-sponsored by the University of North Carolina and Universidad de San Francisco de Quito. Human subjects ethics approval for this research project was obtained from both universities.
7.3. Measures

**Immune Function Biomarkers:** A minimally invasive finger-stick was used on the 3rd or 4th fingertip to draw approximately 50µL of blood collected on protein saver cards (Whatman 903). Samples were allowed to dry and then stored in freezers at the Galapágos Science Center. Eluted blood spots were analyzed for high-sensitivity C-reactive protein (CRP) and endotoxin core IgG antibodies (EndoCab IgG) using commercially available enzyme-linked immunosorbent assays.

Quantikine’s Human C-reactive protein/CRP immunoassay (R&D Systems, Inc. Minneapolis, MN) was used to quantify levels of CRP, as a marker of systemic inflammation, within the range of .003-13 mg/L and a coefficient of variation of 10% across assays. Due to high intra-individual variability of CRP in this pathogenic environment, two measures were used for this study, one at baseline and another after approximately ten days. Median values are reported for measures of association due to the positively skewed distribution and logarithmic transformations were performed for modeling. To provide context in relation to other studies, prevalence and tests of association are reported for acute inflammation indicated by the clinical cut point of over 10mg/L and an additional measure of above 3mg/L change between the two time points, previously found to better indicate an acute phase response to infection. Only logarithmic transformed continuous models are used for hypothesis testing.

Hycult’s EndoCAb IgG ELISA kit (Hycult Biotech Inc. Plymouth, PA) was used to quantify levels of endotoxin core immunoglobulin-G antibodies as an indicator endotoxemia and immunostimulation caused by the translocation of endotoxins across a compromised intestinal barrier, characteristic of EED (Keusch et al. 2014; Korpe and Petri 2012). This composite measure provides the IgG response to lipopolysaccharides from four gram negative bacteria:
Escherichia coli, Klebsiella aerogenes, Pseudomonas aeruginosa, and Salmonella typhimurium (Barclay 1995). Since there are no standard units of EndoCAb or established cut-point for EED, levels are expressed in median units (MU) per mL standardized as a percentage from the median of 1,000 healthy adults (Barclay 1995; Hycult year not specified). The range for the kits was 13-800 MU/mL, with a coefficient of variation of 14% across assays. Since EndoCAb IgG is a long-term marker of “cumulative” EED risk (Benzoni et al. 2015) and intra-individual variability is minimal, only baseline measures are used in this analysis. As with CRP, medians are reported for measures of association and logarithmic transformations are used for the final models. However, prevalence and tests of associations are reported for the percentage of children over the 75th percentile of the total sample indicating elevated levels, as standard cut-points are not available.

E. coli Exposure from Household Water: A sample of 100mL of water was collected from non-drinking water sources in each household at baseline. The most common source was the kitchen or bathroom faucets. However, 8% of households did not have piped water inside the home, and samples were taken directly from the cistern or holding tank. Samples were stored in an insulated bag with icepacks for transport and analyzed daily for levels of E. coli using Colilert’s reagents and Quanti-tray 2000 most probable number (MPN) method (IDEXX Laboratories, Inc. Westbrook, Maine). Although the World Health Organization (WHO) recommends zero MPN E. coli per 100mL of water for drinking (Havelaar et al. 2001), this standard is not easily attainable in rural Ecuador, much like other low and middle income countries (Levy et al. 2012). Therefore, levels were dichotomized as above or below two separate thresholds of 10 MPN E. coli per 100mL and 100 MPN per 100mL as developed by Moe and colleagues (1991) for both tests of associations and statistical models.
**Infection Status:** Mothers were asked whether their children experienced infectious symptoms of diarrhea, vomiting or fever within the last week at baseline and during the second blood spot collection. If one or more symptoms were reported, the child is considered to have an infection for this analysis.

**Obesity:** Standing height and weight were measured using a portable stadiometer and digital scale. Body mass index (BMI) was calculated using weight(kg)/height(m)² and transformed into z-scores using WHO reference data (de Onis et al. 2007; WHO 2006). Although the extent to which overnutrition may influence intestinal inflammation and barrier function is unclear, CRP is greatly affected by obesity (Ford et al. 2003) and statistical models are adjusted for obesity using a BMI z-score over two.

**Other Measures:** Highest socio-economic status (SES) was determined by land ownership elsewhere on the island as 47% of households own their homes (CGREG and INEC 2010). Other measures such as maternal education and employment, and ethnicity were explored and found to have no significant effect in statistical models. Since baseline data were collected over two periods less than one year apart, field season is included to adjust statistical models for possible sampling biases or seasonal effects.

7.4. **Statistical Analyses**

A mix of parametric and non-parametric tests were used to determine significant differences between the presence of infectious symptoms and *E. coli* levels for CRP and EndoCAb measures. Differences in age groups were also explored as both CRP and EndoCAb levels are age dependent (Barclay 1995; Dowd et al. 2010). Since exposure to fecal pathogens may cause diarrhea and gastrointestinal illness (Elliott 2007), we further tested for significant
associations between infection status and *E. coli* levels of over 10 and over 100 bacteria per mL, separately.

Mixed effects linear models were used to test the effects of *E. coli* levels and infection on log-transformed CRP while adjusting for intra-individual variability between the two time points and ordinary least squares regression was used to test the effects on log-transformed EndoC Ab. The estimates and standard errors were adjusted for clustering at the household level for both types of models. Both *E. coli* thresholds were modeled separately with infection status and no other covariates to determine which *E. coli* level to use in the fully adjusted model with other covariates. To test the hypothesis that children with infections and low *E. coli* exposure have the highest levels of immunostimulation, fully adjusted models with the interaction variable between *E. coli* exposure and infection status were used to calculate the predicted exponentiated estimates of CRP and EndoCab, while holding covariates at their observed values.

### 7.5. Results

**Sample Characteristics:** Summary statistics are described for the total sample in Table 7.1. Approximately 48% of the sample were recruited and interviewed during the first field season and slightly more than half (52%) of the 116 children were boys. The mean age was 5.7 (SD 2.6) years and 19% were considered obese. Ten children came from households that own private property elsewhere on the island, indicating the highest socio-economic status. Mothers reported infectious symptoms in 36% of the children. The mean *E. coli* level in household non-drinking water samples was 128 (SD 277) MPN per 100mL of water, with 42% of households having over 10 MPN *E. coli* per 100mL and 12% having over 100 MPN *E. coli* per 100mL.

**Associations with Age, Reported Infections, *E. coli* Levels on Immune Function and the Relationship between *E. coli* and Reported Infections:** Table 7.2 summarizes key outcome
measures for CRP and EndoCAb. No significant differences were found between sexes and age groups in median CRP levels at both time points. Older children had significantly higher EndoCAb levels than younger (154 vs. 134MU/ml, respectively; p<0.01). Children with reported infections had higher CRP levels, though the difference was not significant. Yet, 30% of children with a change of CRP levels 3mg/L or greater between time points reported infections, compared to only 4% without infectious symptoms (p<0.01). EndoCAb levels were also higher in children with infections (150 vs. 144MU/mL), but the difference was not significant. No significant differences in children with elevated EndoCAb levels were found. Children from households with high *E. coli* levels over 10 bacteria reported a greater percentage of infections than children from households with lower bacteria levels (44% vs. 30%).

CRP levels were significantly lower at both time points among children from households with *E. coli* levels over 10 MPN per 100mL compared to under 10 MPN bacteria (time-1: 0.26 vs. 0.46mg/L, time-2: 0.27 vs. 0.43mg/L, respectively; p<0.05). The median CRP level at time-2 was even lower among households with *E. coli* over 100 MPN per 100ml at 0.11mg/L, also statistically significant compared to children from household with below 100 MPN *E. coli* (p<0.01). The median EndoCAb level among children from households with over 10 MPN *E. coli* per 100mL water was slightly higher than from under 10 MPN bacteria (150 vs. 142MU/mL, respectively); however, children with over 100 MPN *E. coli* had the lowest median level of 134 MPN yet not significant. All children with *E. coli* levels over 10 MPN per 100mL water had EndoCAb levels over the healthy adult median, compared to 84% with *E. coli* levels under 10 MPN (p<0.01).

**Unadjusted and Adjusted Covariate Models:** In the unadjusted models, *E. coli* levels over 10 MPN per 100mL had a significant negative effect on log-transformed CRP (β -0.59,
while only controlling for infection status ($\beta$ 0.48, $p=0.010$) (Table 7.3). *E. coli* levels over 100 MPN had a similar negative effect ($\beta$ -0.56, $p=0.155$) but was not statistically significant and thus *E. coli* over 10 MPN was used in the fully adjusted CRP covariate model. The negative effect of *E. coli* over 10 MPN per 100mL ($\beta$ -0.52, $p=0.034$) and opposing positive effect of reported infectious symptoms ($\beta$ 0.52, $p=0.003$) on log-transformed CRP held while adjusting for field season, sex, age, SES, and obesity. However, obesity is the only other covariate to have a significant effect of log-CRP, which is slightly stronger in increasing log-CRP ($\beta$ 0.90, $p=0.000$) than infection.

Contrary to the unadjusted log-transformed CRP models, the higher *E. coli* threshold of over 100 MPN per 100ml water had a significant negative effect on log-transformed EndoCAb ($\beta$ -0.12, $p=0.027$) while only adjusting for infection ($\beta$ 0.05, $p=0.368$) (Table 7.4). *E. coli* over 10 had a slight negative effect ($\beta$ -0.01, $p=0.905$), yet was not significant and not used in the full model. Similar to the effects of high *E. coli* levels on log-CRP, *E. coli* over 100 has a significant negative effect on log-EndoCAb ($\beta$ -0.12, $p=0.016$), while the opposing positive effect of infection was not significant ($\beta$ 0.04, $p=0.409$). Age was the only other covariate to have a significant effect ($\beta$ 0.03, $p=0.003$), which is consistent with the bivariate associations.

**Interactions Models:** Table 7.5 summarizes the effects for the interaction terms where children with infections and low *E. coli* levels are the referent, while fully adjusting for covariates (coefficients not shown). In the log-transformed CRP model, among children with infections, we found a significant protective effect of high *E. coli* exposure ($\beta$ -0.76, $p=0.016$) compared to low *E. coli* levels (referent). As expected, the effect of having no infection regardless of high or low *E. coli* exposure has a negative relationship with CRP. The negative effect size of no infection and high *E. coli* ($\beta$ -1.04, $p=0.000$) is greater than no infection with
low *E. coli* exposure (β -0.73, p=0.002). All three interactions statistically lower CRP levels compared to infection with low *E. coli*. This suggests that children with infections and low *E. coli* exposure levels have the highest immune stimulation, compared to children with infections and the protective effect of high *E. coli* exposure levels, or not having an infection. The mean predicted exponentiated CRP levels are plotted for each group using the interaction model in Figure 7.1. Children with infection and low *E. coli* levels have the highest predicted CRP levels (1.00mg/L, SD 0.44), compared to children with infections and high *E. coli* levels (0.37mg/L, SD 0.13) or children without infections. Remarkably, the protective effect of high *E. coli* exposure lowers CRP levels 0.10mg/L more in children with infections than healthy children with low *E. coli* exposure (see the two inside bars in Figure 7.1).

In the interaction model for log-transformed EndoCAb, the three interactions have similar negative effects on EndoCAb compared to the effect of children with infections and low *E. coli* exposure, yet only infections with high *E. coli* levels was significant. Among children with infections, we found a significant protective effect of high *E. coli* levels (β –0.20, p=0.003) on log-EndoCAb. However, as demonstrated in the fully adjusted covariate model, there are no protective benefits in lowering log-EndoCAb levels by the absence of infection. Figure 7.2 displays the mean predicted exponentiated EndoCAb values by the interaction terms. Children with low *E. coli* exposure had higher predicted EndoCAb levels compared to high *E. coli* exposure, regardless of the presence of infectious symptoms. However, unlike the lower predicted CRP levels of children without infection, EndoCAb levels are only slightly affected by infection status. Children with low *E. coli* exposure and infection have the highest predicted EndoCAb levels at 150MU/mL (SD 13), followed by children with low *E. coli* levels and no infection at 141MU/mL (SD 10).
7.6. Discussion

As hypothesized, we found that higher *E. coli* exposure from non-drinking water sources related to daily hygiene and sanitation practices provided protection from immune activation of EED biomarkers in children living Galapágos, Ecuador. Median CRP levels were more than 37% lower among children with higher *E. coli* exposure levels, compared to children with lower *E. coli* exposure, and the median EndoCAb levels were lowest among children with the highest exposures. Our adjusted covariate models showed negative relationships between *E. coli* levels in household water and both CRP and EndoCAb levels, suggesting a protective effect of daily, low-grade pathogenic exposure on inflammatory and antibody-mediated immunostimulation among these children. These findings complicate the common assumption that chronic exposure to fecal contaminated water is the driving factor in EED, providing possible evidence of the immunoregulatory benefits of habitual, mild *E. coli* exposure that does not result in direct intestinal infection.

Acquired immunity could explain why we do not see higher levels of fecal contamination increasing immunostimulation associated with EED or infectious symptoms of diarrhea and vomiting. Children can acquire immunity to the pathogens found in their own feces and those of people living in the same household, which are likely to contaminate surfaces, floors and stored household water. An observational study of fecal contamination among squatter community households in Lima, Peru proposed that a child will acquire immunity to enteropathogens after they are initially exposed to a mild dose that does not cause clinical infection or diarrhea, but is able to break through the gastric barrier into the small intestine (Lanata et al. 1998). Similarly, chronic exposure to existing fecal coliforms commonly found in contaminated household water may not produce an immune challenge. Some strains of pathogenic *E. coli* (including attaching
and effacing *E. coli* and heat-labile toxin strain of enterotoxigenic *E. coli*) have been shown to provide protection against diarrhea caused by reinfection among Guinean (Valentiner-Branth et al. 2003) and Mexican infants (Cravioto et al. 1990). Among children in the Cebu Longitudinal Health and Nutrition Survey, in-house fecal coliform contamination of household drinking water did not predict episodes of diarrhea, while external contamination of novel coliforms did increase risk of diarrhea (Vanderslice and Briscoe 1993). Among Cebu children without diarrhea, 21% had enteropathogens present in fecal swabs indicating acquired immunity.

Although we found that households with higher levels of *E. coli* reported more children with diarrhea, vomiting and fever, than households with lower bacteria levels, these differences were not statistically significant. Evidence of a causal link between ingesting fecal pathogens in contaminated drinking water and childhood diarrhea is inconsistent (Gundry et al. 2004). For example, weekly assessments over a year-long period of *E. coli* levels in public and household drinking water and childhood diarrhea in Pakistan revealed no significant relationship (Jensen et al. 2004). In Bangladesh, aggregated household monthly diarrhea rates were marginally associated with average *E. coli* concentrations in wells where household obtained drinking water (Escamilla et al. 2013). Some studies have demonstrated a direct relationship between fecal contamination of water sources and childhood diarrhea, while adjusting for other community and household sanitation practices (e.g., (VanDerslice and Briscoe 1995)), which may be due to exposure to new or higher doses of enteropathogens, such as viruses or protozoa, or simply other unidentified sources of contamination. Levy and colleagues (2012) have cautioned that environmental, host and pathogen considerations are vital in choosing which fecal indicator to use in representing exposure to enteropathogens, since they are commonly found in tropical climates. When comparing a variety of human fecal indicator organisms to detect water
contamination in relation to diarrhea episodes in coastal Ecuador, *E. coli* demonstrated the highest performance and reliability (Levy et al. 2012). However, all these complicating factors do not explain the strong significant protective effect of *E. coli* in lowering levels of immunostimulation.

Our study found a strong protective effect of fecal pathogen exposure on lowering immunostimulation associated with EED, even when interacting exposure with the presence of infectious symptoms. In support of our hypothesis, children with infections and low *E. coli* exposure had the highest predicted CRP and EndoCAb levels, compared to all other interactions. Even among children without reported infectious symptoms of diarrhea or vomiting, higher *E. coli* exposure was significantly associated with lower CRP levels compared to low *E. coli* exposure. The most notable evidence of this protective effect of high fecal contamination exposure was that children with infectious symptoms who experienced high *E. coli* exposure had lower predicted CRP levels than children without infectious symptoms who experienced low *E. coli* exposure. Since habitual ingestion of fecal contaminated water is assumed to cause diarrhea and EED (Korpe and Petri 2012; Watanabe and Petri 2016), we would have expected to find that children with infections and high *E. coli* exposure would have the highest levels of immunostimulation. Infection status does not lower EndoCAb levels, which was anticipated since it is a subclinical condition. Yet higher *E. coli* exposure does lower predicted EndoCAb compared to lower *E. coli* exposure, which is significant among children with diarrhea and vomiting. Our results provide novel support for the “old friends” hypothesis that early life exposure to environmental immunoregulatory microbes help develop and regulate the immune system.
Our study contributes to this evolutionary perspective by 1) the use of a direct, empirical measure of *E. coli* contamination in household non-drinking water, 2) the immunoregulatory effect of early life exposure to fecal pathogens is demonstrated in lowering both predicted levels of inflammation (indicated by high-sensitive CRP) and the humoral response to endotoxins (indicated by EndoCAb IgG antibodies), and 3) this effect is present during early and middle childhood (ages 2-10 years). Rook and colleagues (2014) propose that the regulation and resolution of the inflammatory response are vital to proper immune functioning as inflammation causes damage to the host, can disrupt commensal microbial colonization and is energetically very costly. Common exposures to environmental “old friend” microbes in mammalian evolutionary past evolved to develop an immunoregulatory influence on the human immune system. Living conditions and lifestyle changes typical of high-income countries limit exposure to these “old friends” in soil, contaminated water and with contact to other animals. Lack of exposure to these pathogens early in life cause inadequate immune development, dysbiosis in the gut microbiota and inflammatory dysregulation, all leading to higher rates of autoimmune and chronic inflammatory disorders (Rook et al. 2013). In a longitudinal prospective study from the Philippines, increased frequency of diarrhea and exposure to animal feces during infancy significantly lowered the predicted CRP levels in young adults (McDade et al. 2010). McDade (2012) hypothesized that higher microbial exposures during infancy, typical of environmental living conditions in low and middle income countries, properly initiate inflammatory responses resulting in more episodes of the acute phase reaction and thus improving inflammatory networks and resolution later in life. This was further supported by the absence of low-grade, chronic inflammation found among an indigenous adults with a high infectious disease burden from the Ecuadorian Amazon (McDade et al. 2012b). To this date, only one other study has
shown a protective effect of the presence of *E. coli* on the hands of care-givers and in drinking water on lowering the risk of diarrhea among from Tanzanian children (Mattioli et al. 2014).

Could *E. coli* be responsible for this immunoregulatory effect? *E. coli* has great genetic and phenotypic diversity resulting in both pathogenic and commensal strains, varying in virulence factors and their ability to provide protection against other bacterial overgrowth (Figler and Dudley 2016; Leimbach et al. 2013). *E. coli* is one of the first anaerobic bacteria to colonize the infant gut shortly after birth and is highly adapted to the mucosal lining of the intestines (Leimbach et al. 2013). Some *E. coli* strains have virulence factor genes, coding for fimbriae that carry adhesins to attach to mannose-receptors found on intestinal epithelial cells, facilitating rapid colonization of the gut (Wold et al. 1988). While most strains are transient only residing for several days, some can become resident for longer periods of time (Caugant et al. 1981). Exposure to higher levels of *E. coli* in fecal contaminated water provides a paradoxical situation that can both increase risk of ingestion of pathogenic strains, possibly causing illness, and commensal strains, providing resistance to pathogenic overgrowth.

Several commensal *E. coli* strains have received much attention for their ability to prevent and treat clinical infection and diarrhea in human and animal models. In a double-blind clinical trial, newborn infants who received the Nissle 1916 strain within the first few days of life had significantly lower incidence and diversity of pathogenic *E. coli* and other enteropathogens at age six months (Lodinová-Žádniková and Sonnenborn 1997). In another trial, the probiotic Nissle 1917 stopped acute diarrhea due to nonspecific infection faster than placebo among children aged two to four (Henker et al. 2007). Similarly, infants colonized with the commensal *E. coli* strains (O83, O86 and Nissle 1917) experienced enhanced humoral and cellular immune responses with elevations in *E. coli*-specific IgA and non-specific polyclonal IgM antibodies,
compared to placebo-controls (Cukrowska et al. 2002; Lodinova-Zadnikova et al. 1991). These studies suggest that early colonization of commensal *E. coli* initiates the proliferation of lymphocytes in general, and multi-reactive, polyclonal antibodies with the ability to provide early life protection against pathogenic *E. coli* and a diverse array of other pathogenic exposures. Exposure to commensal *E. coli* among children in our study could be providing a protective immunoregulatory effect.

In addition to immunoregulation, commensal *E. coli* and even some pathogenic strains have genetic virulence factors that provide defensive mechanisms against other enteropathogens. Commensal *E. coli* strains are highly antagonistic and Nissle 1917 has shown to outcompete Salmonella through nutrient requisition in mice (Deriu et al. 2013). Similarly, Nissle 1917 outcompetes enterohemorrhagic *E. coli* by adhering to epithelial cells and reducing shiga-like toxins in vitro (Rund et al. 2013). The EMO strain has even been shown to outcompete pathogenic, drug-resistant *E. coli* in newborn human infants inoculated at birth (Duval-Iflah et al. 1983). Some *E. coli* strains, J02 and J03, have the ability to produce microcin, small bacteriocin that inhibit the growth of similar Gram-negative bacteria (Portrait et al. 1999). The commensal *E. coli* EMO strain can provide a barrier effect against enterotoxigenic strains (which typically cause traveler’s diarrhea) in mice and pig models (Duval-Iflah et al. 1983). In other mice models with and without the inflammatory colitis, exposure to the Nissle 1917 increased zonula occludens-1 gene expression, strengthening epithelial tight junctions and providing protection against poor intestinal barrier function (Ukena et al. 2007). In mice models with lipopolysaccharide-induced sepsis, Nissle 1917 produced a systemic anti-inflammatory effect by reducing T cell cytokines and IgG antibodies (Arribas et al. 2009). Exposure to *E. coli* in fecal contaminated water is a possible candidate providing the protective effect in our study given its
abilities to outcompete other pathogens, initiate an anti-inflammatory responses and improve intestinal barrier function. However, *E. coli* are indicator bacteria for fecal contamination and many other pathogens, including viruses, protozoa and helminths, present in the water may be contributing to this immunoregulatory effect. Comprehensive microbial analysis of water quality is needed, in addition to investigating genetic variation of *E. coli* strains, to determine which combination of protective mechanisms are at work in our study sample.

We found no evidence of a significant relationship between levels of inflammation and the antibody response to endotoxins in our sample (CRP time-1 & EndoCAb r=0.15; CRP time-2 & EndoCAb r=0.02). Similarly, a recent study of EED in Bangladeshi infants demonstrated weak correlations between systemic inflammation with lactulose: mannitol (L:M) levels indicating EED, and with biomarkers for intestinal inflammation (Campbell et al. 2017). Among these infants, 60% demonstrated signs of systemic inflammation (CRP>5mg/L or AGP>100mg/dL), yet only 39% had EED (indicated by L:M>0.07). These results suggests that EED may not be the primary driver of elevated levels of systemic inflammation among these Bangladeshi infants. However, our study found higher levels of immunostimulation associated with EED and lower levels of systemic inflammation (5% had CRP>10mg/L at time-1 or -2) among Galápagos children. A direct comparison of the prevalence of EED cannot be determined since the EndoCAb IgG biomarker does not have clinical cut-points and changes to the assay over time make population comparisons somewhat problematic. However, the Galápagos children aged two to five years demonstrated higher mean EndoCAb levels than children of similar ages from both Malawi (Benzoni et al. 2015) and Bangladesh (Lin et al. 2013). We propose that variations in gut microbiome caused by nutritional and pathogenic environments may provide a protective effect against systemic inflammation caused by endotoxemia.
Our study was limited in number of ways, such as the use of one fecal indicator bacteria, *E. coli*, to measure exposure to fecal contamination. We were unable perform advanced microbial testing to determine what other fecal pathogens were present in the water and contributing to the immunoregulatory effects in children. Similarly, we were unable to do genetic testing to determine which commensal or pathogenic *E. coli* strains were present, nor microbial source tracking to identify the source of contamination. However, a concurrent water study on the island concluded that the *E. coli* present in the household tap water was likely from environmental or animal sources and not from human waste (Gerhard et al. 2016). In addition, only a single measure of *E. coli* exposure was used, although longitudinal water quality measures were collected from a sub-sample to evaluate intra-household variability over time. No statistically significant differences were found in *E. coli* levels taken on average ten days apart from 82 households, suggesting that our measure reflects chronic exposure and not acute elevations. Invasive biopsies are necessary for clinical diagnosis of EED and no single set of EED biomarkers are universally used (Syed et al. 2016). Nonetheless, EndoCAb IgG is among the most commonly used, as lactulose: mannitol ratios have recently found to be uncorrelated with immune measures (Campbell et al. 2017). The limitations of using EndoCAb antibodies are that there are no standard units of measurement and thus assay levels are standardized around the mean of healthy adults (Barclay 1995), even when levels are taken from children. Furthermore, no clinical cut-points for EED exist, and changes to the assay development make between population comparisons challenging (Kosek et al. 2014). Lastly, our study is observational in design and we cannot confirm causal links between exposures and immune levels, nor were we able to capture all exogenous factors.
7.7. Conclusion

Using a quantitative measure of fecal pathogens in household non-drinking water, our study is the first to our knowledge to empirically test the assumption that habitual, low-grade exposure to \textit{E. coli} contaminated water impacts immunostimulation associated with EED among children in a dual burden environment. Contrary to clinical and epidemiological hypotheses, our results support the developmental “old friends” theory that early life exposure to some pathogens common in our evolutionary past provide an immunoregulatory effect in helping to initiate pro- and anti-inflammatory pathways. We expand on previous studies to demonstrate that \textit{E. coli} is possibly an immunoregulatory pathogen with protective effects on lowering inflammation and enhancing intestinal barrier function in early and middle childhood. Our study differs from other investigations of EED in that the Galapágos children have access to clean drinking water and are experiencing overnutrition. Exposure to fecal contaminated water is primarily from daily hygiene behaviors and household sanitary practices, such as brushing teeth, washing hands, bathing, cleaning dishes and rinsing raw fruits and vegetables. In addition, the Galapágos children may be experiencing resource buffering covers the energetic cost of immunocompetence. Since the gut microbiome is a vital immunomodulator that can influence mucosal immunity, intestinal health and systemic immune function (Hand 2016; Kau et al. 2011), we propose that symbiotic changes to gut microbiota caused by nutritional and pathogenic environments may be underlying the protective effect of \textit{E. coli} exposure on lowering immunostimulation among these children from Galapágos, Ecuador and future analysis of the gut microbial data from our sample will elucidate this hypothesis.
Figure 7.1 Mean predicted exponentiated CRP levels.

Figure 7.2 Mean predicted exponentiated EndoCAb levels.
Table 7.1 Sample characteristics

<table>
<thead>
<tr>
<th></th>
<th>Total Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>166</td>
</tr>
<tr>
<td>First Field Season</td>
<td>80 (48%)</td>
</tr>
<tr>
<td>Boys</td>
<td>86 (52%)</td>
</tr>
<tr>
<td>Mean Age</td>
<td>5.7 (SD 2.6)</td>
</tr>
<tr>
<td>BMI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.4 (SD 2.6)</td>
</tr>
<tr>
<td>BMI z-score &gt;2</td>
<td>31 (19%)</td>
</tr>
<tr>
<td>Highest SES&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 (6%)</td>
</tr>
<tr>
<td>Infectious Symptoms</td>
<td>60 (36%)</td>
</tr>
</tbody>
</table>

Mean MPN<sup>c</sup>  
*E. coli* per 100mL  128 (SD 277)  
*E. coli* >10 MPN per 100mL  70 (42%)  
*E. coli* >100 MPN per 100mL  19 (11%)

<sup>a</sup> body mass index  
<sup>b</sup> social economic status  
<sup>c</sup> most probable number  
SD standard deviation
### Table 7.2: Associations with age, reported infections and E. coli exposure levels on immune function biomarkers and infectious symptoms

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ages</th>
<th>No Infection</th>
<th>Infection</th>
<th>E. coli &lt;10 MPN per 100mL</th>
<th>E. coli &gt;10 MPN per 100mL</th>
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<td></td>
<td>2 - 4.9</td>
<td>Sample</td>
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<td>Presence of Infections</td>
<td>CRP &gt;10mg/L at either time</td>
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<td>31</td>
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<td>150</td>
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<td>150</td>
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<td>150</td>
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</table>

*p < 0.05; **p < 0.01; for differences between age groups, infection status, E. coli levels <10 MPN per 100mL, E. coli levels >100 MPN per 100mL
<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>SE</th>
<th>P</th>
<th></th>
<th>β</th>
<th>SE</th>
<th>P</th>
<th></th>
<th>β</th>
<th>SE</th>
<th>P</th>
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<td>0.19</td>
<td>0.010</td>
<td>*</td>
<td>0.397</td>
<td>0.19</td>
<td>0.039</td>
<td>*</td>
<td>0.516</td>
<td>0.18</td>
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<td>E. coli &gt;10 MPN per 100mL</td>
<td>0.599</td>
<td>0.21</td>
<td>0.005</td>
<td>**</td>
<td>-0.322</td>
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<td>0.520</td>
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<td>-0.397</td>
<td>0.30</td>
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<td>0.497</td>
<td>0.31</td>
<td>0.055</td>
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<tr>
<td>First field season</td>
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<td>0.25</td>
<td>0.905</td>
<td></td>
<td>0.397</td>
<td>0.30</td>
<td>0.155</td>
<td></td>
<td>0.497</td>
<td>0.31</td>
<td>0.055</td>
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<tr>
<td>Sex</td>
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<td>0.16</td>
<td>0.128</td>
<td></td>
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<td>0.037</td>
<td>0.788</td>
<td></td>
<td>0.375</td>
<td>0.34</td>
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<tr>
<td>Age</td>
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<td>0.788</td>
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<td>0.273</td>
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<td>Highest SES</td>
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<td>0.788</td>
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<td>0.375</td>
<td>0.34</td>
<td>0.273</td>
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* p<0.05; ** p<0.01

Unadjusted for covariates
Adjusted for covariates

Table 7.3: Fixed effects of log-CRP models
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<th>Infection</th>
<th>unadjusted log (-\text{EndoCAb})</th>
<th>adjusted log (-\text{EndoCAb})</th>
<th>(\beta)</th>
<th>SE</th>
<th>P value</th>
<th>(\beta)</th>
<th>SE</th>
<th>P value</th>
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<tr>
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<td></td>
<td></td>
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<td>E. coli &gt;10 MPN per 100mL</td>
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<td>0.054</td>
<td>0.379</td>
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<td>0.053</td>
<td>0.368</td>
<td>0.0447</td>
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Table 7.4: Coefficients of interaction model for log-CRP and log-EndoCAb models.
CHAPTER 8. PAPER 3- GUT MICROBIOTA MEDIATE IMMUNEOSTIMULATION

Gut microbial symbiosis underlying the protective effect of fecal pathogen exposure on immunostimulation association with environmental enteric dysfunction

8.1. Introduction

Chronic immunostimulation caused by environmental enteric dysfunction is known to contribute to childhood stunting in the developing countries due to unhygienic living conditions and malnutrition (Campbell et al. 2003a; Humphrey 2009; Mondal et al. 2012; Prendergast et al. 2014; Solomons 2003). Environmental enteric dysfunction (EED) is a subclinical condition of the small intestines characterized by chronic, local inflammation that compromises intestinal barrier function, allowing for the translocation of endotoxins into the bloodstream initiating endotoxemia and systemic immune responses (Korpe and Petri 2012; Watanabe and Petri 2016). Our previous research revealed an unexpected protective effect of exposure to moderate levels of fecal pathogens in contaminated household (non-drinking) water sources on inflammation and endotoxemia associated with EED (Figure 8.1, top). Contrary to epidemiological theory, this work provided novel support of the “old friends” mechanism that suggests that modern lifestyles, improved living conditions and environmental changes may have limited our exposure to key immunoregulatory pathogens in water, soil and with contact to animals from our evolutionary past (Rook et al. 2014). The lack of these exposures inhibits the proper development and regulation of the human immune system in early life, hindering one of its essential functions of resolving inflammation following infection. Our findings are in agreement with work by McDade and colleagues (2010) that found exposure to animal feces and episodes of diarrhea during infancy lowered inflammation levels in early adulthood, and with other studies.
documenting the early life effects of exposure to animals in preventing auto-immune disorders such as asthma and allergies (Ege et al. 2011; Waser et al. 2005). Researchers have mainly focused on the cellular pathways, such as increased production of regulatory T cells, in explaining this effect (Rook et al. 2017; Yazdanbakhsh et al. 2002). Given that the gut microbiome and its metabolites are important immunomodulators that can influence regulatory T cells and help promote intestinal health (Brown et al. 2013; Hand 2016; Kau et al. 2011), this paper will test whether changes in the gut microbial composition associated with fecal pathogen exposure provide immunoregulatory benefits associated with inflammation and endotoxemia.

The human immune system is an adaptation for negotiating interactions with microbes. Humans and their ancestors have coevolved with microbes throughout our evolutionary history. Roughly 60% of the human genome was derived during the beginnings of life with the evolution of prokaryote and eukaryote cells (Domazet-Lošo and Tautz 2008). These shared genes have facilitated relationships with bacteria and eukaryote single-celled microbes, which led to the development of the adaptive immune system (Rook et al. 2017). Through the operation of natural selection on the host and microbe levels, commensal intestinal bacteria developed symbiotic functions with the human diet to breakdown plant carbohydrates, synthesize vitamins and regulate immune function (O’Hara and Shanahan 2006). Gut microbiota are responsible for nutrient metabolism, such as digesting dietary fiber and producing short chain fatty acid (SCFA) metabolites. SCFAs provide key signals to the inflammatory immune response and are responsible for maintaining the intestinal epithelium and barrier function (Kau et al. 2011; Maslowski and Mackay 2011). Pathogenic and dietary environments, along with genetic and other host factors, can influence microbial symbiosis providing for normal immunoregulation, or can disrupt the microbial balance leading to dysbiosis, immune dysregulation and chronic
inflammation (Levy et al. 2017; Liddicoat et al. 2016; Maslowski and Mackay 2011). For example, when stimulated by *Escherichia coli* and other pathogenic bacteria, microbial compositions can cause a pro-inflammatory immune phenotype due to specific metabolites that control levels of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) (Schirmer et al. 2016). Although it has been suggested that chronic ingestion of fecal pathogens may cause gut microbial dysbiosis, leading to chronic inflammation, bacterial translocation and endotoxemia characteristic of EED (Brown et al. 2015; Kau et al. 2011), this pathway remains untested in population studies. A recent study of Malawian infants found that the abundance of the phylum Proteobacteria, along with other species level changes, were inversely associated with severity of EED, indicated by the lactulose: mannitol (L:M) sugar absorption test (Ordiz et al. 2017). However, the L:M ratio test’s use and ability to predict immunostimulation associated with EED has been questioned (Campbell et al. 2017; Denno et al. 2014).

We propose that symbiotic modification of the gut microbiome is a mechanism underlying the link between high *E. coli* exposure in household tap water and lower levels of C-reactive protein (CRP), as an indicator of inflammation, and endotoxin core immunoglobulin-G antibodies (EndoCAb IgG), indicating endotoxemia, in children from Galápagos, Ecuador (Figure 8.1, bottom). Exposure to high levels of exogenous fecal pathogens could cause beneficial changes (symbiosis) to the gut microbiome, decreasing harmful gut bacteria and increasing protective bacteria in healthy children without infectious symptoms. Gastrointestinal infections causing diarrhea and intestinal inflammation significantly reduce gut microbial diversity and allow for pathogenic overgrowth (Lupp et al. 2007); therefore, we stratified our sample into children with and without infectious symptoms. Among sick children, these intestinal bacteria may have a different functional role in altering immune function. To test our
hypothesis and identify which gut microbial taxa are responsible for the protective effect of fecal
pathogen exposure from contaminated water, we examine the gut microbial colonies of 105
healthy and 54 sick children aged two to ten years. The first step in our analytic strategy is
identifying which intestinal taxa are significantly influenced by exogenous fecal pathogen
exposure by estimating the odds ratios of elevated gut taxa and determining their statistical
significance (Figure 8.1, middle). Then using only gut taxa that are statistically influenced by
high fecal pathogen exposure in water, the second step is to test whether these gut microbial taxa
are significantly associated with continuous levels of CRP and EndoCAb in healthy and sick
children, separately. Finally, we identify candidate gut taxa for the independent protective effects
on inflammation and endotoxemia if: A) exogenous fecal exposure increased odds of microbial
taxa that are associated with lower immunostimulation, or B) exogenous fecal exposure lowered
odds of microbial taxa that are associated with higher immunostimulation (Figure 8.1, bottom).

8.2. Sample and Data Collection

For this analysis, we sampled 159 children aged two to ten years living on the island of
San Cristóbal, Galápagos, Ecuador. In-depth interviews were conducted with each mother or
primary caregiver to obtain detailed information on the demographics, health and illnesses, and
dietary patterns of the children. One point-of-use tap water sample was collected from each
household to measure levels of fecal pathogen exposure. Two dried blood spots were collected
from each child and assayed for levels of inflammation and endotoxemia associated with
environmental enteric dysfunction. We conducted both water and blood analyses in the
microbiology laboratory at the Galápagos Science Center. Each child contributed one fecal
sample that was analyzed to determine gut microbial composition by the Microbiome Core
Facility at the University of North Carolina, Chapel Hill (UNC). This study received approval for
human subject research by the UNC and the local review board at the *Universidad de San Francisco de Quito*.

### 8.3. Measures

**Fecal Pathogen Exposure:** A 100mL household tap water sample was collected at baseline and quantified for levels of *E. coli* and total coliforms using Colilert reagents and the Quanti-tray 2000’s most probable number (MPN) methodology (IDEXX Laboratories, Inc. Westbrook, MA). Bacteria levels ranged from zero to the upper detection limit of 2,420 MPN for both *E. coli* and total coliforms. Based on the distribution for this analysis, children in households above the 75th percentile of the MPN for the total sample of *E. coli* or total coliforms, independently, were considered to have high fecal pathogen exposure. We assume that these bacteria levels represent chronic exposure to fecal pathogens and do not reflect acute fluctuations in water quality. To test this assumption, we re-tested water samples from 82 households taken on average ten days after baseline. We classified levels at both time points based on the World Health Organization’s (WHO) health risk categories for *E. coli* levels in drinking water (per 100mL): low risk (<1 MPN), intermediate risk (1-10 MPN), high risk (10-100 MPN), very high risk (>100 MPN) (WHO 2011b). Approximately 70% of households experienced no change in classification, 17% changed by one level, and 13% changed by two or three levels. We then re-tested 27 households again on average 132 days after baseline and found that paired t-tests using a 95% confidence level indicated no significant differences in bacteria levels between baseline and ten days, or baseline and 132 days for *E. coli* or total coliforms.

**Gut Microbial Composition:** Mothers were given detailed instructions for the collection and storage of fecal samples during the baseline interview. The fecal samples were collected within one week of the initial interview and processed and stored in freezers at the Galápagos
Science Center until fieldwork was completed. Samples were then transported to the Microbiome Core Facility at UNC for analysis. DNA isolation was performed using protocols in the Qiagen BioRobot Universal (Qiagen, Valencia, CA) and quantified using Quant-iT™ PicoGreen® dsRNA Reagent (Molecular Probes, Life Technologies division of Thermo Fisher Scientific, Waltham, MA). 16S rDNA bacterial amplicon pyrosequencing was performed on a Roche GS FLX Titanium instrument (Microbiome Core Facility, UNC Chapel Hill, NC). Sequencing data was analyzed using the QIIME pipeline (Caporaso et al. 2010) and assigned into operational taxonomic units (OTUs). We chose to analyze microbial compositions at the family level because it allows for a broad array of bacterial types without being too extensive. Family level OTUs above the total sample prevalence 0.35% were used to limit the analyses to bacteria contributing minimal proportion. For each child, the abundance of the top 26 family taxa were calculated as OTU percentages. A child is considered to have a relative elevated abundance of any specific family taxa if it was over the 75th percentile for the total sample. This arbitrary cut-point was chosen based on the distribution of the data as it represents the highest quartile of the sample.

**Immunostimulation:** Dried blood spots were collected at baseline and after approximately ten days. Around 50µL of blood were drawn from each child using a minimally invasive finger-stick on the 3rd or 4th fingertip and collected on specialized protein saver cards (Whatman 903). Cards were sealed and stored in freezers at the Galápagos Science Center until fieldwork was completed. Eluted blood spots were analyzed using enzyme-linked immunosorbent assays (ELISA) to quantify levels of immunostimulation associated with environmental enteric dysfunction. Quantikine’s Human C-reactive protein/CRP immunoassays (R&D Systems, Inc. Minneapolis, MN) were used to determine levels of high-sensitivity C-
reactive protein (CRP) as an indicator of inflammation. Hycult’s EndoCAb IgG ELISA kits were used to quantify levels of endotoxin core immunoglobulin-G antibodies (EndoCAb IgG) as an indicator of endotoxemia. For statistical analyses, logarithmic transformations of CRP and EndoCAb levels were performed. Since intra-individual variability in CRP levels is high in environments with heavy disease burdens (McDade et al. 2012a), two time points were used for modeling. The single baseline measure of EndoCAb was used for models as intra-individual variability is low and it is considered a stable measure of cumulative EED risk (Benzoni et al. 2015).

**Infectious Status:** During the baseline interview and during the second collection of blood spots, mothers were asked whether their children experienced diarrhea, vomiting or fever within the past week. In this analysis, children were considered to be healthy if no infectious symptoms were reported or sick if any one symptom was reported.

**Other Covariates:** Weight and standing height were measured using a portable scale and stadiometer for each child. Body mass index was calculated by weight(kg)/height(m)² and converted to z-scores using the WHO’s references data (de Onis et al. 2007; WHO 2006). Children were considered obese if BMI-for-age z-scores were above two. Models were adjusted for obesity, age and sex.

**8.4. Statistical Analyses**

Difference in sample characteristics between healthy and sick children were explored using t-tests and Fisher’s exact tests. To examine the gut family taxa distribution for the entire sample, we calculated the mean percentage of the family taxa abundance, stratified by the presence/absence of infectious symptoms. We used t-tests to explore differences in gut microbial abundance by infection status. To determine the influence of exogenous fecal pathogen exposure
on gut family taxa abundance, we used logistic regression to estimate the odds ratios of high *E. coli* and total coliforms exposure, independently, on elevated family taxa abundance while adjusting for age, sex and infections status (Figure 8.1, middle). Each of the 26 gut family taxa were modeled separately, and taxa yielding statistically significant results with a 90% confidence level were chosen for the next analytical step. To test for the effects of the microbial families influencing immunostimulation, we standardized the percent abundance of each of the selected family taxa to allow for comparisons between effect sizes of the family taxa on immune levels. We used mixed effects linear models of log-transformed CRP, adjusted for intra-individual variability between the two CRP measures, to determine the effects on inflammation. Ordinary least squares models of log-transformed EndoCAb were used to examine the effects on endotoxemia. Since gut microbial composition can be a cause and consequence of gastrointestinal infection, the sample was stratified by present/absence of infectious symptoms. Each microbial family taxa predictor was modeled separately for healthy and sick children, adjusting for age and sex, and obesity in the CRP models. Sandwich estimators were used to adjust for clustering at the household level. We selected microbial families as candidates for the protective effect of exogenous fecal pathogen exposure on immunostimulation associated with environmental enteric dysfunction if they met one of the following criteria: A) fecal exposures were related to higher odds of elevated taxa that were associated with lowered immune levels, or B) fecal exposures were related to lower odds of elevated taxa that were associated increased immune levels (Figure 8.1, bottom).

### 8.5. Results

Approximately 34% of children experienced infectious symptoms of either diarrhea, fever or vomiting within the past two weeks (Table 8.1). The mean *E. coli* level in household
drinking water was 86 MPN per 100mL water and the mean total coliform level was 1,302 MPN per 100mL. Although sick children lived in households experiencing higher mean levels of *E. coli* and total coliforms, these differences were not statistically significant. Mean CRP levels for children with infectious symptoms were twice as high as healthy children at baseline; yet EndoCAb levels were not significantly different.

Microbial composition of the top 26 abundant family taxa for the total sample are listed in Table 8.2. Three of the top five: *Ruminococcaceae, Lachnospiraceae* and *Erysipelotrichaceae*, belong to the phylum Firmicutes. *Coriobacteriaceae* is from the Actinobacteria phylum and *Bacteroidaceae* belongs to Bacteroidetes. We found significant differences in the abundance of *Ruminococcaceae, Streptococcaceae* and *Actinomycetaceae* between children with and without infectious symptoms.

Odds ratios for elevated family taxa that were significantly (p<0.1) influenced by either high *E. coli* or total coliform exposures are reported in Figure 8.2 and are adjusted for age, sex, infection status and clustering at the household levels (covariates are not shown). High *E. coli* exposure was associated with significantly higher odds of elevated *Bacteroidaceae* (OR 2.46, 95%CI 1.01-6.00) and *Clostridiaceae* (OR 2.55, 95%CI 1.01-6.47), and marginally higher odds of elevated *Porphyromonadaceae* (OR 2.07, 95%CI 0.90-4.78). Whereas *E. coli* exposure was related to lower odds of high levels of *Bifidobacteriaceae* (OR 0.41, 95%CI 0.16-1.04). High total coliforms were shown to lower odds of elevated *Bifidobacteriaceae* (OR 0.45, 95%CI 0.20-.098), *Enterobacteriaceae* (OR 0.43, 95%CI 0.18-1.02) and *Alcaligenaceae* (OR 0.46, 95%CI 0.22-0.97).

The standardized effects of selected family taxa on levels of inflammation and endotoxemia were fully adjusted for age, sex and clustering at household level, and obesity in the
CRP models (Figure 8.3). Beta coefficients are interpreted as the effect on log-transformed CRP and EndoCAb levels resulting from one standard deviation change in family taxa abundance. Both *Bacteroidaceae* (β -0.10, SE 0.06, p=0.099) and *Bifidobacteriaceae* (β -0.013, SE 0.07, p=0.041) were associated with marginally lower CRP levels in children with infections. In healthy children, *Porphyromonadaceae* was associated with reduced CRP levels (β -0.09, SE 0.05, p=.051). *Enterobacteriaceae* was related to substantial elevated CRP levels among all children, regardless of infection status (healthy β 0.26, SE 0.04 p=0.000; sick β 0.33, SE 0.01, p=0.001). Contrary to the effect on inflammation, *Bacteroidaceae* (β 0.07, SE 0.01, p=0.000) was related to substantial increases in EndoCAb level among sick children. *Alcaligenaceae* (β 0.03, SE 0.01, p=0.043) was shown to increase levels of EndoCAb and *Clostridiaceae* (β -0.04, SE 0.02, p=0.029) was shown to greatly reduce levels in healthy children.

Based on our criteria for selecting family taxa responsible for the protective effect of fecal pathogen exposure on immunostimulation, we determined that *Enterobacteriaceae* was the strongest candidate for inflammation in both healthy and sick children (Figure 8.4). High total coliform exposure significantly lowered odds of having elevated *Enterobacteriaceae* levels, which increased CRP levels regardless of infection status. Alternatively, fecal bacterial exposure increased *Bacteroidaceae* and *Porphyromonadaceae*, which both slightly reduced CRP levels in children with infectious symptoms and without, respectively. However, their effects on lowering inflammation were only marginally significant (p<0.1). We found two equally likely candidates for the protective effect on endotoxemia in healthy children. High *E. coli* exposure increased the odds of elevated *Clostridiaceae* abundance, which reduced EndoCAb levels, while high coliform exposure decreased odds of elevated *Alcaligenaceae*, which increased levels of EndoCAb. No
family taxa met the criteria for a protective effect on endotoxemia among children with infectious symptoms.

8.6. Discussion

Our study provides novel evidence that compositional symbiotic differences in the gut microbiome may be an underlying mechanism of the “old friends” hypothesis, linking early life pathogenic exposures to immune health and protection from environmental enteric dysfunction. We found support for our hypothesis that changes in the gut microbiome associated with exposure to high fecal pathogens in household tap water provide immunoregulatory effects, lowering levels of inflammation and endotoxemia among healthy and sick children from Galapágos, Ecuador. High *E. coli* and total coliform exposures significantly impacted the odds ratios of elevated levels of six family taxa, out of the 26 most abundant in the study sample. *E. coli* and total coliforms exposures were associated with the abundance of families with the ability to influence measures of immunostimulation used in our study. To our knowledge, this is the first study to test the impact of empirical measures of fecal pathogen exposure from contaminated water on the gut microbiome and to determine the influence of microbiota on immunostimulation associated with environmental enteric dysfunction in children. We suggest that these differences contribute to the maintenance of gut symbiosis, or the balance of commensal bacteria allowing for proper immune regulation, clearing of pathogenic infection and resolution of inflammation (Levy et al. 2017).

We determined that *Enterobacteriaceae* is the most likely candidate for the protective effect of fecal pathogens on CRP levels, signifying enhanced resolution of the inflammatory response (Figure 8.1, bottom: pathway B). *Enterobacteriaceae* was related to significantly higher CRP levels in our models, which was expected given about 10-20% of common gastrointestinal
infections in children are caused by bacteria from this family, such as *E. coli*, *Shigella*, *Salmonella* and *Yersinia enterocolitica* (Elliott 2007). However, high fecal pathogen exposure was associated with lower odds of elevated abundance of the pro-inflammatory *Enterobacteriaceae*. The fact that we observed a negative relationship between total coliforms and *Enterobacteriaceae* was unexpected since fecal coliforms also belong to this family. Unlike rare, acute exposures to fecal pathogens in contaminated food resulting in gut dysbiosis, diarrhea and vomiting, we suggest that chronic exposure to fecal pathogens from household water sources may over time entrain the gut to resist pathogenic overgrowth from this family. Antagonism between commensal and pathogenic bacteria for nutrients and adhesion to the intestinal epithelium prevent pathogenic overgrowth (Henker et al. 2007). Children exposed to higher levels may have commensal microbiota with the ability to outcompete pathogenic bacteria and initiate rapid immune responses, thus lowering total amounts pathogenic species in the gut. *Enterobacteriaceae* comprised less 0.47% of total bacterial composition in this sample (Table 8.2); yet given it demonstrated the strongest effect size for increasing levels of CRP in children with and without infectious symptoms, small changes in the abundance could have significant impacts on immunostimulation. Local inflammation caused by gastrointestinal infection promoted the overgrowth of *Enterobacteriaceae*, especially nonpathogenic *E. coli*, replacing bacterial species of Firmicutes in mice models (Lupp et al. 2007). However after the infection was cleared, initial bacteria levels were restored indicating rapid resolution of inflammatory responses and the ability to reestablish microbial homeostasis following diarrheal infection.

While the family level *Enterobacteriaceae* collectively was related to higher levels of inflammation and had no effect on endotoxemia levels in our sample, other studies have demonstrated that commensal *E. coli* strains provide enhanced antibody immune responses
(Lodinova-Zadnikova et al. 1992) and some anti-inflammatory effects by inhibiting pro-inflammatory cytokines (Arribas et al. 2009). In a study of the gut microbiota and their associated endotoxins or lipopolysaccharides (LPS) in Eastern European and Russian infants, Vatanen and colleagues (2016) found that *E. coli* was one of the dominant bacteria contributing to the biosynthesis of lipid-A, which is key component of the LPS molecule that signals the innate immune response. The *E. coli* LPS-subtype produced high levels of pro-inflammatory cytokines TNFα and interleukin-1β (IL-1β), the anti-inflammatory IL-10, and IL-6, in addition to providing resistance to immunostimulation after re-exposure to endotoxins. This signifies that *E. coli* is a vital immunoregulatory pathogen that trains the innate immune system and can provide endotoxin tolerance in early life. However, the study also demonstrated that the relatively high contribution of *Bacteroides* species to the gut microbial composition prevented and blocked the protective cytokine response of co-occurring *E. coli* LPS-subtype (Vatanen et al. 2016). Since our sample is older in age and therefore has more abundant *Bacteroidaceae* bacteria (Koenig et al. 2011), the regulatory properties of *E. coli* on inflammation and endotoxemia may have been suppressed.

Our study revealed opposing effects of *Bacteroidaceae* on immunostimulation. It was marginally associated with reduced systemic inflammation and higher levels of endotoxemia in children with infectious symptoms. *Bacteroides fragilis* has shown to decrease LPS-induced inflammatory cytokines and chemokines during childhood when colonized during early infancy (Sjogren et al. 2009). Although the authors speculate this suppressive effect may be due to endotoxin tolerance, other research indicates that *Bacteroides* LPS-subtypes failed to lower concentrations of TNFα after re-stimulation, signifying inability to produce endotoxin tolerance (Vatanen et al. 2016). Mice with elevated abundance of *B. fragilis* experienced higher levels of
microbial translocation and endotoxemia (Romond et al. 2008). Gambian infants with elevated amounts of *Bacteroidaceae* also experienced abnormally high levels of fecal calprotectin (Davis et al. 2017), suggesting intestinal inflammation characteristic of environmental enteric dysfunction (Keusch et al. 2014). In mice models, exposure to a mixture of several Bacteroidales species and *E. coli*, along with a malnourished diet, induced structural and functional changes to the small intestines mimicking human environmental enteric dysfunction (Brown et al. 2015). Bacteria belong to the *Bacteroidaceae* family may have differential abilities to increase risk of intestinal inflammation causing compromised barrier function and translocation of endotoxins into the bloodstream inducing endotoxemia, while others provide immunoregulatory effects on systemic inflammatory cytokines signaling.

We identified *Clostridiaceae* and *Alcaligenaceae* as candidate bacterial families for providing endotoxin tolerance among healthy children, indicated by reducing endotoxin core IgG antibodies. *Clostridiaceae* was associated with a significant protective effect in lowering levels of endotoxemia, and *E. coli* was related to a higher odds ratio of elevated abundance. In mice models, helminth infection has been shown to increase the expansion Clostridiales colonization, which provided protection from some bacterial overgrowth through stimulation of Type 2 Helper T cells (Ramanan et al. 2016). Commensal bacteria strains from *Clostridia* have been found to have heighten immunoregulatory effects in promoting regulatory T cells and anti-inflammatory IL-10 isolated from human fecal samples, and have reduced abundance in patients with inflammatory bowel disease (Atarashi et al. 2013). In mice models with the inflammatory disorders of colitis and allergic diarrhea, administration of these *Clostridia* strains attenuated symptoms and reduced humoral and inflammatory responses (Atarashi et al. 2013). Thus, bacteria from *Clostridiaceae* exhibit immunoregulatory benefits in regards to reducing intestinal
inflammation under chronic conditions that are consistent with our findings of lowering endotoxemia levels induced by intestinal inflammation. However, our study is the first to our knowledge to identify the family Alcaligenaceae as inducing immunostimulation or being influenced by fecal pathogen exposures. We found no candidate microbial family for the protective effect on endotoxemia in sick children, which may be explained by the fact that environmental enteric dysfunction that causes endotoxemia is a subclinical condition. Thus, it is not associated with diarrhea or the other infectious symptoms we used to indicate sick children in our study. There is no theoretical reasoning behind stratifying childhood EndoCAb levels by infection status, other than methodological consistency with our analysis of CRP that is dependent infection status.

Bifidobacteriaceae was related to decreased inflammation levels among sick children from our study. Human milk oligosaccharides are bioactive molecules found in breastmilk that help establish the colonization of the commensal genus Bifidobacterium and Bacteroides, which are their primary consumers (Marcobal and Sonnenburg 2012; Sela and Mills 2010). Bifidobacteria nourished by human milk oligosaccharides have been shown to improve immunoregulatory effects by increasing anti-inflammatory cytokine IL-10 and strengthened epithelial tight junctions by increasing levels of junctional adhesion molecule A (Chichlowski et al. 2012). Infants who are exclusively breastfed demonstrate higher proportions of Bifidobacterium and lower proportions of Bacteroidetes and Clostridiales than non-exclusively breastfed or formula fed infants (Thompson et al. 2015). When complimentary foods are given with age, the proportion of Bifidobacterium declines and Bacteroides increases (Koenig et al. 2011), and thus children in our sample, aged two to ten years no longer have a dominance of Bifidobacterium (Table 8.2). However, the protective effects of lowering inflammation and
endotoxemia (although not statistically significant in our models) are experienced through adulthood. Oral administration of a commensal strain, *Bifidobacteria infantis*, for eight weeks significantly lowered levels of CRP and pro-inflammatory cytokines TNFα and IL-6 in patients with three chronic conditions (ulcerative colitis, chronic fatigue syndrome and psoriasis) and in healthy adults following LPS-stimulation (Groeger et al. 2013). Some *Bifidobacteria* strains increased metabolic production of the short-chain fatty acid, acetate, which enhances epithelial cells and intestinal barrier function, protecting against microbial translocation following exposure to enterohemorrhagic *E. coli* in mice (Fukuda et al. 2011). While *Bifidobacteriaceae* was not considered a candidate mechanism for the protective effect of fecal pathogen exposure since high *E. coli* and total coliform were associated with reduced abundance, our results revealed important immunoregulatory effects supported by other experimental studies.

Although we establish that the presence of infectious symptoms and chronic fecal pathogen exposure in contaminated household water impacts the gut microbial composition in children while adjusting for demographics, our models were limited in capturing other factors impacting gut symbiosis. Birth delivery method (Dominguez-Bello et al. 2010), infant feeding practices (Koenig et al. 2011), antibiotic use (Jernberg et al. 2010) and long-term dietary patterns (De Filippo et al. 2010; Wu et al. 2011b) can influence gut microbiota. Data of delivery type was not collected for our entire sample; however, 47% of 135 children were born by cesarean section. Approximately 35% of children in our sample were exclusively breastfeed for six months, with a much higher percentage actually receiving breastmilk in combination with formula. To determine the effect of breastfeeding, we ran additional models adjusting and stratifying for infant feeding practices that yielded similar results and thus were excluded. Antibiotic or antiviral use were not included as only 3% of children in our study received these medications.
Environmental enteric dysfunction causes structural changes in the small intestine and microbial compositions analyzed from fecal samples in this study may not provide a regional representation of colonization specific to that area (Brown et al. 2015). While our interpretations assume causal relationships between fecal pathogen exposures, bacterial family abundance and immune function, our analytic design does not test for causality, and it is unknown whether family taxa increase immunostimulation or whether immune function can manipulate bacterial abundance. The effect of individual diets on microbial compositions remains unexplored in our sample; however based on ethnographic observation, children consumed high-fat, low-fiber diets typical of populations experiencing nutrition transition (Popkin et al. 2012). Other studies have demonstrated this dietary pattern is associated with higher levels of Bacteroides than Prevotella (Wu et al. 2011a). This finding is supported by a greater abundance of Bacteroidaceae compared to Prevotellaceae in our total sample and a high prevalence of childhood obesity. Future research will examine the relationships between diets, microbial compositions and immune function in our sample.

8.7. Conclusion

To the best of our knowledge, this is the first observational human health study to demonstrate that variation in the gut microbiome is a mechanism of adaption to chronic exposure to fecal pathogens that increases fitness by providing immunoregulatory effects. Previous studies have documented the many immunomodulatory properties of the gut microbiome in relation to intestinal health, symbiosis and systemic infection. However, these studies have been mostly experimental in design, performed in animal models, or only concerned with the impact of microbiota on immunostimulation and do not reflect on how the specific compositions are influenced by the pathogenic, dietary and physical environments in which people live. The
distinction between what is classified as gut immune symbiosis or dysbiosis will be dependent on
the individual’s unique history of microbial exposures and nutrition status. Researchers
previously assumed that habitual fecal exposure from contaminated water and food due to poor
sanitation infrastructure and limited access to clean drinking water was the cause for increased
levels of chronic immunostimulation characteristic environmental enteric dysfunction
(Humphrey 2009; Watanabe and Petri 2016). Our results contradict this assumption and instead
elucidate the mediating role of the gut microbiome and its immunoregulatory capacities. Using
an evolutionary framework, we identified several microbial pathways linking early life fecal
pathogen exposures on immunoregulation related to environmental enteric dysfunction in a
human population model. The practical significance of these results help to better understand
environmental-induced susceptibility to inflammation and endotoxemia in children living in high
pathogenic environments, typical of low and middle income countries.
Observed relationship from previous work

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Analytical strategy

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Step 1. odds ratios of elevated levels

Step 2. significant associations

Effect on Immune Levels

Hypothesized mediating relationship of gut symbiosis

Exogenous fecal pathogen exposure
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect on Immune Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>lower</td>
</tr>
</tbody>
</table>

Figure 8.1 Observed and hypothesized relationships of fecal pathogen exposure on immunostimulation.

Top: High fecal pathogen exposure was associated with lower levels of immunostimulation in previous work. Middle: Step 1) Determine the odds ratios of elevated abundance of gut taxa based on high fecal pathogen exposure. Step 2) Test whether significant gut microbial taxa are associated with immune levels. Bottom: The two pathways for candidate selection of the protective effect of fecal pathogen exposure on immunostimulation: A) High fecal pathogen exposure is associated with increased abundance of beneficial bacteria that lowers immune levels. B) High fecal pathogen exposure is associated with decreased harmful bacteria that elevate immune levels.
Figure 8.2 Odds ratios of fecal pathogen exposure on family taxa.

Note: Estimates are adjusted for age, sex, infection status and clustering at the household level.
Figure 8.3 Standardized effects of family taxa on immunostimulation.

Note: Estimates are adjusted for age, sex, obesity (CRP models) and clustering at the household level.
Table 8.4 Gut microbial candidate selection for the protective effect of fecal pathogens on immunostimulation.

<table>
<thead>
<tr>
<th>Gut Symbiosis</th>
<th>Candidate for EndoCAb</th>
<th>Candidate for CRP</th>
<th>CRP</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysbiosis</td>
<td>+</td>
<td>-</td>
<td>AL3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>Porphyromonadaceae (4%)</td>
<td>+</td>
</tr>
<tr>
<td>Strongest Candidate for CRP</td>
<td>+</td>
<td>-</td>
<td>Porphyromonadaceae (4%)</td>
<td>-</td>
</tr>
<tr>
<td>Coliforms (1.3%)</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae (2.9%)</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candidate for CRP</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroidaceae (3.5%)</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 8.4** Gut microbial candidate selection for the protective effect of fecal pathogens on immunostimulation.
### Table 8.1: Sample characteristics, fecal pathogen exposure and levels of immunostimulation

<table>
<thead>
<tr>
<th></th>
<th>Total Sample</th>
<th>Healthy</th>
<th>Sick</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>159</td>
<td>105 (66%)</td>
<td>54 (34%)</td>
</tr>
<tr>
<td>Male</td>
<td>83 (52%)</td>
<td>53 (50%)</td>
<td>24 (44%)</td>
</tr>
<tr>
<td>Mean Age</td>
<td>5.7 (SD 2.6)</td>
<td>5.8 (SD 2.5)</td>
<td>5.6 (SD 2.6)</td>
</tr>
<tr>
<td>Obeseᵃ</td>
<td>27 (17%)</td>
<td>18 (17%)</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>Presence of Infectious Symptoms</td>
<td>-</td>
<td>-</td>
<td>54 (34%)</td>
</tr>
<tr>
<td>Infection</td>
<td>9 (17%)</td>
<td>18 (17%)</td>
<td>27 (17%)</td>
</tr>
<tr>
<td>Total Sample</td>
<td>110 (SD 377)</td>
<td>74 (SD 211)</td>
<td>86 (SD 278)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean (MPN per 100mL)</th>
<th>75th percentile (MPN per 100mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal Expose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>86 (SD 278)</td>
<td>55</td>
</tr>
<tr>
<td>E. coli 75th percentile</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Mean E. coli Levels</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean (CRP level (mg/L))</th>
<th>Mean EndoCAb level (MU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunostimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>1.3 (SD 2.7)</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>1189 (SD 1080)</td>
<td>55</td>
</tr>
<tr>
<td>E. coli 75th percentile</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Mean E. coli Levels</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ body mass index - z-score > 2
ᵇ baseline measures

*p*<0.05 Fisher exact
<table>
<thead>
<tr>
<th>Family Taxa</th>
<th>Healthy</th>
<th>Sick</th>
<th>Total Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruminococcaceae</td>
<td>21%</td>
<td>17%</td>
<td>23%</td>
</tr>
<tr>
<td>Lachnospiraceae</td>
<td>15%</td>
<td>16%</td>
<td>15%</td>
</tr>
<tr>
<td>Coriobacteriaceae</td>
<td>6.1%</td>
<td>5.9%</td>
<td>6.6%</td>
</tr>
<tr>
<td>Erysipelotrichaceae</td>
<td>4.7%</td>
<td>4.1%</td>
<td>5.9%</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>3.6%</td>
<td>3.4%</td>
<td>3.8%</td>
</tr>
<tr>
<td>Clostridiaceae</td>
<td>2.9%</td>
<td>2.9%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Streptococcaceae</td>
<td>2.6%</td>
<td>4.0%</td>
<td>1.9%</td>
</tr>
<tr>
<td>Veillonellaceae</td>
<td>2.3%</td>
<td>1.9%</td>
<td>2.5%</td>
</tr>
<tr>
<td>Rikenellaceae</td>
<td>2.2%</td>
<td>2.3%</td>
<td>2.3%</td>
</tr>
<tr>
<td>Prevotellaceae</td>
<td>2.1%</td>
<td>2.5%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Enterococcaceae</td>
<td>1.5%</td>
<td>1.4%</td>
<td>1.9%</td>
</tr>
<tr>
<td>Paraprevotellaceae</td>
<td>1.3%</td>
<td>1.5%</td>
<td>0.99%</td>
</tr>
<tr>
<td>Bifidobacteriaceae</td>
<td>1.3%</td>
<td>1.1%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Mogibacteriaceae</td>
<td>1.1%</td>
<td>1.1%</td>
<td>1.3%</td>
</tr>
<tr>
<td>Barnesiellaceae</td>
<td>1.0%</td>
<td>0.93%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Actinomycetaceae</td>
<td>1.0%</td>
<td>0.70%</td>
<td>1.3%</td>
</tr>
<tr>
<td>Tissierellaceae</td>
<td>0.90%</td>
<td>0.96%</td>
<td>0.69%</td>
</tr>
<tr>
<td>Odoribacteraceae</td>
<td>0.72%</td>
<td>0.73%</td>
<td>0.69%</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>0.59%</td>
<td>0.62%</td>
<td>0.52%</td>
</tr>
<tr>
<td>Aerococcaceae</td>
<td>0.43%</td>
<td>0.42%</td>
<td>0.45%</td>
</tr>
<tr>
<td>Porphyromonadaceae</td>
<td>0.41%</td>
<td>0.46%</td>
<td>0.32%</td>
</tr>
<tr>
<td>Alcaligenaceae</td>
<td>0.38%</td>
<td>0.45%</td>
<td>0.26%</td>
</tr>
<tr>
<td>Pasteurellaceae</td>
<td>0.36%</td>
<td>0.23%</td>
<td>0.62%</td>
</tr>
</tbody>
</table>

*p<0.05, t-test between presence/absence of infections

Table 8.2: Family taxa abundance for the total sample and by infection status.
CHAPTER 9. DISSERTATION SYNTHESIS

9.1. Significance of Galápagos as a Dual Burden Environment Research Setting

Due to economic growth and infrastructure development over the past several decades, lifestyles and health patterns in Galápagos have undergone dramatic transformations. Commerce and tourism have replaced subsistence farming and fishing, leading to a reliance on the importation of food and basic supplies by boat and plane. The dietary shift from local production to processed foods and a lower diversity in fresh produce has created a pattern of high-fat, low-fiber diets consistent with the overnutrition seen in high income countries. In addition to lower physical activity levels, these changes likely explain why Galápagos currently has the highest rates of overweight and obesity among Ecuadorian children and adults. Rapid immigration from the mainland and an increasing number of tourists visiting each year are increasing the strain on the already weak water and sanitation infrastructures. High levels of fecal contamination in the municipal water supply and untreated sewage has generated a living environment with high risk of pathogen exposure and infectious disease among its residents. Understanding the early life health impacts of living in a dual burden environment, where obesity and cardiovascular diseases are coupled with infectious illness, is critical in preventing childhood obesity and gastrointestinal disease, and improving growth outcomes.

9.2. Overall Strengths and Limitations of the Study Design

The strengths of this dissertation were the use of traditional anthropological and evolutionary theories adapted to fit the conditions of the dual burden environment, and the testing of new hypotheses concerning the causes and consequences of the interaction between
immunostimulation from both pathogenic and obesogenic sources. In addition, intestinal health and immune function were measured at three different levels: 1) systemic inflammation measured by C-reactive protein (CRP); 2) poor intestinal barrier reflecting microbial translocation of endotoxins into the blood stream, initiating endotoxemia and the humoral antibody response function indicated by endotoxin core immunoglobulin-G antibodies (EndoCAb IgG); and 3) intestinal symbiosis/dysbiosis evaluated by gut microbial compositions. Incorporating immune biomarkers with data from the gut microbiome is uncommon among other anthropological health studies.

Another strength of this study was the use of longitudinal measures of CRP. To estimate rates of elevated inflammation indicating cardiovascular disease risk in Paper 1, it was vital to examine intra-individual variability over time to determine whether elevations were stable and chronic, or fluctuating in response to infection or injury. To explore the influence of high *Escherichia coli* exposure and gut microbial compositions on inflammation in Papers 2 and 3 respectively, using multiple measures in the statistical models allowed for flexibility in examining positive or negative relationships. This analytic strategy supported the conceptual objective in using the modified life history theory framework for the dual burden environment. Thus, the study was concerned with the effect of increasing or decreasing levels on the inflammatory response, and not determining the impact of predicting chronic, low-grade inflammation or acute inflammation over certain cut-points.

The use of empirical measures to assess water quality for *E. coli* and total coliforms, instead of relying on proxy measures of fecal contamination, is innovative for human health studies in anthropology. In comparison to other water, sanitation and hygiene (WASH) studies, the age range from two to ten years investigated in this research includes the entire span of early
to middle childhood, instead of focusing on children under five. Another strength of this dissertation was the incorporation of a follow-up study after, on average, six and one-half months from recruitment. The purpose of this follow-up was to measure weight gains and linear growth in children, in order to model life history tradeoffs between immunocompetence and growth outcomes that have been presented at national meetings, though not a part of this dissertation.

Rather than examine the mismatch that occurs between changing resource environment during early and later life, this dissertation used the developmental origins of health and disease (DOHaD) framework to explore the early life impacts of simultaneous pathogenic and obesogenic exposures on gut health and immune function.

There are several general limitations of this study. First, the extent to which these results can be generalized to other dual burden populations is unclear. However, this study does provide methods for assessing chronic inflammation and frameworks to explore the impact of poor water quality on immune function and gut health in other similar dual burden populations. In addition, loss to follow-up for the final round of data collection was over 50%, which limited our analysis using linear growth and weight gain measures. The inability to infer causality from the statistical models is problematic for most observational studies, as are unidentified endogenous factors not captured in the analytic design. Limitations concerning measures are discussed below in relation to specific analyses.

9.3. Paper Summaries and Contributions

Paper 1- Measuring Chronic Low-Grade Inflammation examined different methods for measuring chronic, low-grade inflammation using cross-sectional and longitudinal measures of CRP to determine which measure most accurately predicts obesity, while excluding elevations associated with infection. Using sensitivity and specificity analyses and the area under the
receiver-operator characteristic (ROC) curve, this study found that the method of calculating the mean value of longitudinal CRP measures and excluding individuals with a high change had the greatest discriminatory ability in relation to obesity, although statistically similar to cross-sectional measures. The methodological contribution of this work confirms that the common practice of using of a single measure of CRP provides a validate estimate of chronic, low-grade inflammation in this dual burden population, when values over 10mg/L are discarded. However, the results also suggest that when practical, using longitudinal measures to access high levels of change between time points to indicate and discard elevations of infectious origin, are a more consistent and reliable method of estimating inflammation rates associated with obesity.

This work is significant in that there is limited research on the impact of infectious-related elevation of CRP in estimating chronic, low-grade inflammation. Likewise, there are only a few studies that examine intra-individual variability in CRP measures among populations outside of the US, Canada and Europe. Unlike these countries, populations experiencing higher infectious disease burdens may have higher CRP levels due to an inflammatory response from infection or injury, which can lead to overestimated levels of chronic inflammation and cardiovascular disease risk. Prevalence of chronic, low-grade inflammation was estimated based on the methods in combination with ranges used to indicate moderately elevated CRP that revealed the greatest balance between sensitivity, specificity and percent correctly classified. Among Galápagos children, the method of discarding values with a change over 3mg/L and using the relaxed range of 1-10mg/L yielded the prevalence of 20%. According to the cross-sectional measures at time-2 using the range of 3-10mg/L, the prevalence among women was 24%. The limitation of this study is that it was unable to confirm whether the use of the particular method and range signifies cardiovascular disease risk, as the measure was only tested
in relation to obesity. The ideal strategy would have been to test the measures in relation to other inflammatory biomarkers and cardiovascular disease.

**Paper 2- E. coli Exposure and Immune Function** tested the impact of early life habitual exposure to *E. coli* from fecal contaminated household (non-drinking) tap water on gut health and immune function in children, to determine if fecal pathogens may provide immunoregulatory effects. Statistical models indicated that high levels of *E. coli* in tap water were significantly associated with lower levels of both CRP and EndoCAb in children. The theoretical contribution of these findings provide novel support for the evolutionary “old friends” hypothesis suggesting that chronic *E. coli* exposure, which does not directly result in diarrhea or infection, may strengthen anti-inflammatory networks and enhance endotoxin tolerance.

Epidemiological studies that have found high levels of immunostimulation associated with environmental enteric dysfunction (EED) in populations with inadequate sanitation, unsafe drinking water and undernutrition. To my knowledge, this is the first study to identify a possible protective effect of fecal pathogen exposures among children living in a dual burden environment with adequate nutrition. Prior studies have found that indirect measures of pathogen exposure, such as unsanitary household environments, poor hygiene practices and childhood behaviors such as geophagy, increase levels of EED. The methodological strength of this study is the direct, empirical measurement of *E. coli* contamination of household water quality.

The significance of this research is that even in the context of a pro-inflammatory state, driven by overweight and obesity, early life exposure to *E. coli* contaminated (non-drinking) water can provide an immunoregulatory effect among children in Galápagos. Although the regulatory abilities of commensal *E. coli* have been demonstrated in probiotic clinical trials and in experimental animal models, that fact this effect was identified in an observational human
population study is novel. Further research is needed to determine what other fecal pathogens and which E. coli strains were present in the water and responsible for the protective effect. One of the strengths of this study is that it demonstrates that early life pathogen exposures may provide immunoregulatory effects even during childhood. Yet, to make significant contributions to the DOHaD theory, follow-up studies are necessary to test whether these effects persist into later life.

**Paper 3- Gut Microbiota Mediate Immunostimulation** determined whether gut microbial compositions mediate the regulatory impact of fecal pathogen exposure in household tap water on immune function associated with EED. Gut microbial taxa were identified as possible candidates for a protective effect on inflammation and endotoxemia separately. Taxa were selected if exogenous exposure to fecal pathogens provided gut microbial symbiosis. For example, if models indicated fecal exposures were associations with higher levels of gut microbial taxa that lowered immunostimulation, or if models found that fecal exposures lowered levels of gut taxa that raised immunostimulation. The study identified that the gut family of Enterobacteriaceae was the strongest candidate for having a protective effect on inflammation. Both Clostridiaceae and Alcaligenaceae were candidates for having a protective effect on endotoxemia.

Testing and identifying gut microbial symbiosis as a possible mechanism underlying the immunoregulatory effect of early life fecal pathogen exposure on inflammation and endotoxemia is an important contribution to the evolutionary “old friends” hypothesis. The significance of this research is its suggestion that the gut microbiome may allow for phenotypic plasticity in inflammatory profiles and endotoxin tolerance of the humoral immune response, based on local ecologies. Demonstration of this effect within the dual burden environment is particularly
interesting as the gut microbiome can also be highly influenced by obesogenic factors. Models for inflammation in this analysis were adjusted for obesity, yet the interaction with diets and body composition measures remains to be tested. Changes to the gut microbiome associated with ingestion of fecal pathogens, in combination with a malnourished diet, have shown to cause EED in mice models. To my knowledge, this is the first human health study to test the hypothesis that alterations in microbial composition due to exposure to fecal contamination, are associated with immune indicators of endotoxemia. This is of particular importance to public health research on EED since its pathological etiology is poorly understood.

9.4. Directions for Future Research

Since the objective of this dissertation is the investigation of early life pathogenic and obesogenic environments on childhood intestinal health and immune function, incorporating the role of dietary factors would greatly strengthen this analysis. Dietary resources may be buffering the energetic costs of immunostimulation associated with inflammation and endotoxemia on childhood health and growth. This may be the reason why the study results are inconsistent with other research that has found an association between higher pathogenic conditions and indicators of environmental enteric dysfunction (Humphrey 2009). In these malnourished populations, environmental enteric dysfunction is responsible for a moderate proportion of stunting (Campbell et al. 2003a; Mondal et al. 2012; Panter-Brick et al. 2009), since resources are diverted from growth to immune function. It will be important to determine the impact of different diets and the role of food insecurity on immune function measures associated with EED. Children with lower caloric intake may have higher levels of immunostimulation since their energy reserves may not meet the requirements to provide for adequate immunoregulation.
over time. These children may also experience lower weight and height gains, compared to
children with higher caloric intake.

Both long and short term dietary patterns can also highly influence gut microbial
symbiosis and diversity (David et al. 2014; Turnbaugh et al. 2009). Distinctions in microbial
compositions have been found between children with high-fat, low-fiber diets typical of
populations with overnutrition and those of a more traditional low-fat, high-fiber diets (De
Filippo et al. 2010). The gut microbiome are responsible for nutrient metabolism of dietary fiber,
and produce metabolites that have direct influence on cytokine production of the inflammatory
immune response and also maintain intestinal barrier function protecting against endotoxemia
(Kau et al. 2011). Similar to our findings that the gut microbiome may be modifying the
immunoregulatory effect in the “old friends” hypothesis, the influence of dietary patterns need to
be examined as they may also provide an immunoregulatory phenotype. The impact of diet on
immune function, intestinal health and growth will be explored in future analyses.

9.5. Conclusion

This study used the emerging field of the gut microbiome as pathway to investigate the
early life effects of overnutrition and poor water quality on childhood intestinal health and
immune function in Galápagos, Ecuador. Building upon anthropological and evolutionary
theory, this research developed and tested new hypotheses for the dual burden environment that
1) examined the relationship between three measures of gut health and immune function:
inflammation, poor intestinal barrier function (endotoxemia) and microbial symbiosis, 2)
disentangled the use of CRP levels associated with acute infection and chronic low-grade
inflammation using longitudinal measures, 3) identified an immunoregulatory effect of moderate
fecal pathogen exposure, indicated by *E. coli* levels in contaminated household water on
childhood inflammation and endotoxemia, and 4) determined the possible role of gut microbial symbiosis underlying protective effects early life fecal pathogen exposure on immunostimulation. These findings provide novel insight into the early life health impacts of the dual burden environment on childhood intestinal health and immune function.

The methodological contribution of this work confirms that the use of cross-sectional measures of CRP provide a validate estimate of chronic, low-grade inflammation in this dual burden population, when values over 10mg/L are discarded. Yet, longitudinal measures identifying and excluding intra-individual variability in elevations due of infections provide a more reliable method of estimating inflammation associated with obesity. The significance of this research is the finding that even in the context of overnutrition resulting in a pro-inflammatory state, early life exposure to *E. coli* contaminated water, which does not result in diarrhea or infection, can provide an immunoregulatory effect among children in Galápagos, Ecuador. The theoretical contribution of this study in relation to the evolutionary “old friends” hypothesis is that gut microbial symbiosis is a possible mechanism underlying protective effects of fecal pathogens on inflammation and endotoxemia. This suggests that the gut microbiome may allow for phenotypic plasticity in inflammatory profiles and endotoxin tolerance of the humoral immune response, based on local pathogenic ecologies. Demonstration of this effect within the dual burden environment is particularly important as the gut microbiome can also be highly influenced my obesogenic factors. To my knowledge, this is the first human health study to show that alterations in microbial compositions, due to exposure to fecal contaminated water, are associated with immune indicators of endotoxemia. This is of particular importance to public health research on environmental enteric dysfunction since its pathological etiology is poorly understood.
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