GUT MICROBES, ENTEROPATHY AND CHILD GROWTH: THE ROLE OF THE MICROBIOTA IN THE CYCLE OF DIARRHEA AND UNDERNUTRITION IN PERU

by
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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy.

Baltimore, Maryland
May 23, 2018

Abstract

Background. The cycle between diarrhea and undernutrition continues to underlie a vast proportion of under-five mortality and is the primary driver of long-term disability among children living in lower and middle-income countries (LMIC). Interventions aimed at reducing childhood stunting have not achieved desired results, highlighting the need for novel research and strategies to target this problem. There is increasing evidence that the gut microbiota are implicated in growth acquisition and sustaining intestinal barrier integrity in a manner that impacts immunity to and consequences of disease; however, these relationships have not yet been examined in large-scale studies of children living in LMIC.

Objective. To evaluate relationships between the gut microbial community, child growth, diarrhea and enteric infections (*Campylobacter spp)* in a birth cohort of 271 children aged 0-24 months in Iquitos, Peru.

Methods. Analyses were conducted on children participating in the multi-site cohort study entitled 'The Interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development (MAL-ED)**.'** Data were contributed over two years by mothers and children living in a peri-urban riverine community in Amazonian Peru. Regular home-visits were conducted to ascertain anthropometric indices, illness history, and dietary habits. Length-for-age (LAZ) and weight-for-length (WLZ) Z-scores below -2 were used to classify stunting and wasting, respectively. Fecal specimens were collected during routine surveillance visits at monthly intervals (N=6004) and additionally during each maternal report of diarrheal symptoms (N=2436). Culture methods, immunoassays and amplification methods were employed according to a unified MAL-ED protocol to identify a panel of over 40 protozoa, bacteria and viruses of public health importance. Microbiota in fecal samples contributed at 6, 12, 18 and 24 months were analyzed by polymerase chain reactions using primers to identify variable regions of bacterial 16S ribosomal RNA genes at the Gordon Laboratory at Washington University. Members were binned into operational taxonomic units (OTU) sharing ≥97% nucleotide sequence identity, producing bacterial communities differentiated at the species level which were then used to generate metrics of maturity (microbiota-for-age Z score; MAZ), diversity (Shannon, Simpson indices) and richness (CHAO1, Faith's Phylogenetic Diversity). Multivariable regression was used to detect and describe population-averaged associations between microbial metrics, growth acquisition, illness and infection with a generalized estimating equations approach to adjust for within-child correlations over time. Indicator species analysis (ISA) was employed to identify particular gut taxa whose presence and abundance was statistically indicative of phenotypes of interest.

Results. Two-thirds of children (67%) were stunted and 9% of children experienced wasting before their 2nd birthday. Microbial diversity and richness increased significantly with age and weaning, and were suppressed by breastmilk exposure. In the first two years of life, we detected a suggestive relationship between microbial maturity and WLZ, but did not observe evidence of associations between microbial maturity, diversity or richness with LAZ in the full cohort. LAZ at birth was significantly associated with MAZ score throughout follow-up (β =0.10, p=0.012) and children born stunted had significantly lower gut microbial diversity and richness (Shannon β =-0.19, CHAO1 =-9.75; p-values <0.05) from birth to two years of age. In this subgroup, we additionally observed that children weaned before 24m of age experienced significantly pronounced

deficits in microbial diversity and richness acquisition relative to those with continued breastfeeding.

Nearly all children (96%) experienced diarrhea during follow-up. Odds of being severely stunted increased by 8% with each additional diarrheal episode throughout the first two years of life ($OR=1.08$; $p<0.001$). Cumulative diarrheal frequency, duration and severity were associated with significant reductions in microbial indices ($p<0.05$), and we observed evidence of enduring deficits beyond 1m after exposure. Children who were born stunted experienced greater insults to microbial diversity per diarrheal episode than those children who were not (Interaction terms: Shannon $\beta=-0.04$, $p=0.037$; Simpson $\beta=-0.01$, $p=0.032$). Time elapsed since last diarrheal episode was associated with recovery of Shannon ($\beta=0.02$, $p=0.03$) and phylogenetic diversity ($\beta=0.11$, $p<0.01$) and we detected evidence that this regeneration process was significantly slower among severely stunted children. Lower diversity and richness were associated with increased subsequent diarrheal incidence; a 1-unit increase in the Shannon and Simpson's Diversity scales at 6m corresponding to a mean reduction of 1.3 and 3.4 diarrheal episodes from 6-24m of age, respectively.

By two years of age, 251 (93%) of all children in the cohort had *Campylobacter* present in asymptomatic stools, and 221 (82%) experienced infection with diarrhea. Asymptomatic infection was associated with reduced LAZ concurrently and at 3, 6, and 9m thereafter ($\beta=0.02$, $p<0.01$ across all time points). Frequency of *Campylobacter*-positive diarrhea was associated with a concurrent reduction in -0.03 LAZ ($p=0.002$), independently from all-cause diarrhea. Asymptomatic *Campylobacter* infections were associated with changes to the gut microbial environment. Infection was associated with

increased microbial diversity and richness metrics, and we identified 21 taxa indicative of being in the highest or lowest quartile of infection from birth to two years of age. Of these, seven indicator species showed suggestive evidence of a link with LAZ concurrently and 1m thereafter.

Conclusions. This study provided evidence of associations between the gut microbial community, anthropometric indices, and enteric infections in a population of children experiencing the classical cycle of diarrhea and undernutrition. This is the first study to our knowledge to interrogate these pathways longitudinally in a large, representative sample of infants in LMIC. Our findings generate questions regarding the precise causal mechanisms underlying the observed associations, and should inform subsequent efforts to identify specific and actionable targets to interrupt pathways compounding childhood morbidity and mortality in LMIC.

Advisor: Dr. Margaret Kosek

Thesis readers: Drs. David Sack, Cynthia Sears, Lawrence Moulton and David Dowdy

Alternate readers: Drs. Joanne Katz, Marie Diener-West

Acknowledgements

I am profoundly grateful for the time and effort dedicated to this project by so many, predating and throughout my doctoral studies. First and foremost, I'd like to acknowledge and thank the families in Santa Clara de Nanay, La Union, and Santo Tomas who took part in this study. Over several years, mothers and children committed countless hours to providing information, specimens, and valuable insights to the MAL-ED Study and it is to them that this work is dedicated.

To the eminent public health academics and practitioners who conceived of the MAL-ED Study; thank you for your commitment to combatting global health inequities, conceiving of novel research questions, advocating for vulnerable populations, and supporting students to follow in your path. I am grateful to our team in Iquitos, Peru whose diligence and dedication to their community made this work possible: Maribel Paredes Olórtegui, Mery Siguas Salas, Dixner Rengifo Trigoso, Ruth Rodriguez, Angel Mendez Acosta, Zoila Huiñapi Nolorbe, Matilde Bustos Aricara, Marla Judith Aricari Ahuanari, Rosario Huansi Torres, Rosario Natividad Aricara Macuyama, Victoria Lopez Manuyama and a special thank you to the late Leoncio del Aguila Amasifuen. I am humbled by the endless hours they committed to collecting data, conducting lab work, nurturing community relationships, mentoring students, and am personally indebted to them for their extraordinary hospitality and support in Peru. I would like to extend my heartfelt gratitude to Shirley Montalvan Muñoz, and her family Cesar, Cesar Junior and Diego, for welcoming me with such warmth into their home in Iquitos, providing me with a sense of family far away from my own, and taking time to share with me culture (and cuisine!) of Loreto.

I would like to thank our partners at the Gordon Laboratory in Washington University, who have committed a truly extraordinary amount of time and resources to this collaboration and specifically to supporting my development and training as a researcher. I am immensely grateful to Drs. Jeffrey Gordon and Michael Barratt for their belief in this collaboration and their engagement and leadership throughout; and to Dr. Nicholas Griffin for his dedication, accessibility and mentorship in the analysis, interpretation and communication of our findings. I am also grateful to the many faculty who have provided me with opportunities to develop my skills as a researcher at the Bloomberg School: Drs. Sara Bennett, Melinda Munos, Alain Labrique, Christopher Heaney, Subhra Chakraborty, Andrea Ruff, Priya Duggal, John McGready, Laura Caulfield and Susan Sherman. A special thank you to Dr. Joanne Katz, for encouraging me to pursue this degree and providing me with advice and support throughout.

I am especially grateful to my Thesis Advisory Committee. To Dr. Lawrence Moulton for his mentorship and tireless guidance in my analytic pursuits; Larry, it has been a privilege to have the opportunity to decipher some of your more mystifying questions and commentary, and I am a better researcher for every attempt I made at it! To Dr. David Sack for his expertise and inspirational, lifelong commitment to improving child health and survival through tackling enteric diseases. To Dr. Cynthia Sears, for reminding me to think through the clinical implications of the work that we do, and think critically about how it may actually translate to alleviating illness.

I would like to extend my profound thanks to the family of Dr. R Bradley Sack, whose generosity in establishing the R. Bradley Sack Family Scholarship helped me sustain my doctoral training. I am honored to have been selected as a recipient of this award and humbled by the opportunity to participate in upholding the legacy of such a formidable and impassioned researcher of enteric diseases and child survival. This work was additionally supported by the Bill & Melinda Gates Foundation, the Foundation for the National Institutes of Health, and the National Institutes of Health, Fogarty International Center.

It is difficult to communicate my profound gratitude to my advisor, Dr. Margaret Kosek, for setting expectations high and pushing my academic and intellectual limits, while also providing me with unwavering encouragement and support at every turn. You have provided me with an example of how to be a committed, rigorous researcher and maintain meaningful commitments across multiple sectors and settings. To Pablo Yori, my honorary advisor of sorts, this process would have been simply impossible without your advice, support and companionship. Together you have modeled excellence in public health research and practice while also reinforcing in me the value of family and quality of life in the work that we do. I have genuinely enjoyed and become a profoundly better researcher by virtue of brainstorming for hours on end together, whether in your Baltimore offices or on the banks of the River Nanay. I would like to extend this gratitude to the other enthusiastic members of these brainstorming sessions, Ruthly Francois, Francesca Schiaffino Salazar and Josh Colston, without whose input and support none of this work could have been achieved.

Finally, I am sincerely grateful for the love and tolerance exhibited by my close friends and family throughout this process. To my hilarious, inspiring and diligent doctoral cohort—I believe we all know how lucky we were to have one another. My special thank you to Vanessa Oddo, Julie Ruel-Bergeron, Daniel Erchick, and Brian

Wahl, without whom my statistical methods and mental stability would have emerged from this experience considerably less soundly. To my parents, Drs. Nahid Mozaffari and Ramine Rouhani for motivating me to pursue my education above all, and sacrificing their time, resources and often sanity to my academic and personal development always. To my younger brother Saman, who is my best friend and most trusted advisor, and next to whom I somehow still pale in both maturity and intellect. To the many characters from my other lives: Hal, for always fostering my sense of adventure and pushing me to pursue some of the more intimidating opportunities that came my way; my two Han(n)a's (Hanna Jamal, Hana Odeh), one for luring me to this town, and the other for welcoming me into her community once I arrived; Dr. Leila Jamal for easing me through this sometimes daunting personal and academic transition; and Mansour Al-Rumayyan for making these years more fun than a PhD should ever be − I would not have fallen so deeply in love with Baltimore City without you by my side. Finally and above all, this work is dedicated to Hank – who hated epidemiology – but who is with me always.

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Abbreviations and acronyms

aβ	Adjusted β coefficient
CODA	Community Diarrheal Assessment Score
ETEC	Enterotoxigenic *Escherichia coli*
GEMS	Global Enterics Multicenter Study
ISA	Indicator Species Analysis
LAZ	Length-for-age Z-score
LMIC	Lower and middle income countries
MAL-ED	Etiology, Risk Factors, and Interactions of Enteric Infection and Malnutrition and the Consequences for Child Health and Development Study
MAZ	Microbiota-for-age Z-score
MLR	Multiple Linear Regression
NEC	Necrotizing Enterocolitis
OR	Odds Ratio
OTU	Operational Taxonomic Unit(s)
PCR	Polymerase Chain Reaction
PD	Phylogenetic Diversity
RDP	Ribosomal Database Project
rRNA	Ribosomal RNA
SAM	Severe Acute Malnutrition
SD	Standard Deviation
SDI	Shannon Diversity Index
SE	Standard Error
U5	Under-5
WLZ	Weight-for-length Z-score

Chapter 1: Introduction

The intestinal tract constitutes the largest immune interface between the human body and the outer environment, and is responsible for maintaining immunological tolerance to trillions of microbes while recognizing and defending against invading pathogens. The composition and assembly of the dense community of microbiota populating the human gut and its collective genetic make-up, referred to as the microbiome, is increasingly implicated in metabolism and digestion, host defense against pathogens, and maintenance and modulation of immunity. In recent years, the influence of the gut microbial community on these critical functions has been convincingly demonstrated in laboratory settings. Animal models have been used to manipulate the gut bacterial community to induce obesity and undernutrition, influence a range of infectious and chronic inflammatory diseases, and alter antibody responses to challenge with infection and vaccination. A growing body of evidence conducted in humans corroborates these observations, illustrating differences in gut microbial composition according to varying nutritional profiles and chronic and infectious disease histories and outcomes.

These developments are of particular interest to the public health community for several reasons. It is widely accepted that malnutrition, including both over and underweight, blunts immunity to infection, and increased burden of infection is associated with compromised immune responses and growth trajectories thereafter. While the impacts of interactions between infection, immunity and nutrition have been extensively described worldwide, interventions aimed at interrupting this cycle have underwhelmed the global health community and understanding of their precise mechanisms remains incomplete. Consequences of this gap in knowledge for child health and survival are profound, with diarrheal disease and chronic undernutrition continuing to drive a vast proportion of death and long-term disability among children younger than 5 years globally. The possibility that the gut microbial community may be implicated in these relationships is therefore promising for the development of novel interventions in infectious disease prevention and management, vaccine development, and nutritional support to avert this burden in a range of developmental and epidemiologic settings.

This line of enquiry, while encouraging, remains in a nascent stage in the pipeline of public health research. In lower income settings and particularly among young children, data from human studies is limited. The majority of research has been concentrated in adults living in high-income settings, with large-scale studies in Europe and the USA generating enthusiasm for the use of microbiome data to inform personalized medicine. Comparative lack of research in vulnerable communities and lower and middle-income countries (LMIC) risks compounding existing inequities in access to novel approaches to improving health and quality of life. Where studies *have* been conducted in this demographic, they have been predominantly cross-sectional and limited by extremely small sample sizes. There remains a lack of consensus on what constitutes a healthy versus unfavorable gut community, and how this can be compared across populations that differ vastly in their dietary and environment exposures. Studies with larger and more representative samples are needed to provide a more complete description of population-level behaviors and exposures influencing microbial succession in the gut community of infants and young children, and the potential consequences of different microbial profiles for child health and growth. Specifically, longitudinal studies to illuminate the mechanisms and temporality of proposed relationships between the gut microbiota, enteric infections, immune responses and growth trajectories will be crucial to generating and testing hypotheses. Advancement of these enquiries from experimental laboratory models to epidemiologic studies at the population level will be critical to understanding whether and how this field may yield therapeutic interventions for child survival and quality of life in LMIC.

This dissertation aims to help address these gaps by exploring associations between the bacterial environment of the developing infant gut and indicators of child growth, enteric infection and diarrheal illness in a large birth cohort in the Peruvian Amazon. The following chapters will provide a summary of existing literature informing this work; a theoretical framework illustrating the potential role of the gut microbiota in the cycle of enteropathy and undernutrition; and three distinct sets of analyses interrogating relationships between gut microbial diversity and composition, growth acquisition, and enteric infection and disease from birth to two years of age. We believe that using a large, community-based longitudinal cohort to further characterize these relationships will be an important contribution to the goal of better understanding, and ultimately improving interventions to promote health and growth during a crucial window for child survival and development.

Chapter 2: Background & Rationale

The case of the leaky bucket: interactions between undernutrition, immunity and infection in young children

Despite recent reductions in child mortality, the ongoing toll of the cycle between diarrhea and undernutrition on child health and survival remains staggering. Diarrhea is estimated to directly account for the loss of up to 700,000 among children under 5 years of age (U5) worldwide (1,2). Furthermore, it is estimated that maternal and child undernutrition cause over 3 million child deaths, accounting for nearly half of all U5 mortality annually (3). Not reflected in these global mortality estimates is extent of morbidity and loss of human potential arising from the burden of repeated enteric infections (4). Indeed, experts have recently estimated diarrheal disease to be the leading cause of death and long-term disability among children under 5 years of age worldwide, arguing that focus on mortality and short-term morbidity has substantially and systematically underestimated the profound consequences of repeated diarrheal disease for intestinal function, child growth and overall developmental potential (5,6). New estimates accounting for these sequelae increased the number of disability-adjusted life-years lost to diarrhea by nearly 40% among children under 5, to over 55 million annually (5).

Interaction between undernutrition and infection was first formally described in a seminal paper by Nevin Scrimshaw, Carl Taylor and John Gordon in 1959 (7). During the decades that followed, ongoing work by Scrimshaw and others provided convincing evidence of synergistic interactions between undernutrition and infection, illustrating that the additive effects of the two conditions were more detrimental to human health than the sum of their separate consequences (8). An analogy of a leaky bucket was used to describe the efforts of pouring nutritional support into individuals whose infection profiles were concurrently resulting in nutritional losses; and as a result, calls were made for integrated interventions to account for these interactions in improving health (8,9).

It is difficult to imagine a better example of the leaky bucket than the cycle between undernutrition and enteric infections, specifically. It is now widely accepted that diarrhea impairs growth, and undernutrition is linked to increased incidence, duration, and severity of diarrhea. Moreover, there are consequences of repeated or chronic enteric infections, even in the absence of diarrheal symptoms, for intestinal function and subsequent gains in growth. This section will summarize what is known about how enteric infections and undernutrition exacerbate one another to provide context for the research questions addressed in this study.

Diarrhea and enteric infections impair growth. Monitoring of illness history and growth curves among children in Guatemala provided convincing evidence of the link between diarrhea and enteric infections and growth faltering in the 1960s (10). Subsequent work across African, South Asian and Latin American settings (11–19) confirmed that frequency and duration of diarrheal disease is associated with reductions in weight and height gains, particularly during early childhood when growth trajectories are most plastic. Beyond acute symptomatic disease, murine models and human studies have demonstrated growth suppression associated with periods of infection that occur in the absence of or endure well beyond diarrheal symptoms (20–24). Diarrhea is estimated to be the single most important infectious disease driving undernutrition (3), accounting for up to 25% of stunting worldwide (25). However, these estimates do not include the nutritional consequences of chronic or repeated enteric infections without overt diarrheal symptoms, suggesting that they underestimate the true contribution of these conditions to impaired growth worldwide.

Undernutrition affects immunity to disease. Undernutrition, in turn, increases vulnerability to infectious diseases and their consequences. Initial observations of this relationship described links between protein deficiency, or kwashiorkor, with decreased antibody formation in infants and young children (26). A large body of research conducted since has described various pathways through which both protein-energy malnutrition (PEM) and micronutrient deficiencies (MND) impair immune development and function. PEM affects innate and adaptive immune responses by depleting lymphocyte stores, reducing T-cell differentiation and antibody production, and diminishing both complement proteins and inflammatory cytokines during infection (27). The relationship between MND and immunity is illustrated by Vitamin A deficiency, which is strongly linked to increased infection and child mortality. Vitamin A is now understood to play a key role in maintaining epithelial and mucosal barriers, a crucial, first-line component of immunity, and in promoting T- and B-cell signaling and proliferation necessary for mounting adaptive responses (26–30). Other micronutrients with demonstrated links to immune function include Vitamin E, which improves vaccine responses by promoting T-cell function; and zinc, which has been shown to reduce severity and duration of diarrheal disease (4,26–28).

Evidence of compromised defenses against infectious diseases, including malaria, tuberculosis, diarrheal disease, pneumonia, HIV, and measles, highlights the extent of childhood morbidity and mortality driven by underlying undernutrition (3,26). The most consistent literature illustrating this link has been generated in the field of enteric disease; numerous studies conducted in both clinical and community-based settings have consistently identified child growth deficits as risk factors for incidence, severity, duration and survival rates of infection with enteropathogens of public health importance (31–38).

Consequences for child health & development. Taken together, these studies provide convincing evidence of the synergistic, cyclical relationships between enteric infection, compromised immunity, and undernutrition. But why does this matter?

These interactions give rise to and exacerbate inequities in health and economic opportunity worldwide. Risk factors for undernutrition and enteric infections - such as food insecurity, poor access to water and sanitation resources, and vulnerability to climate events- disproportionately affect the world's poorest and most vulnerable populations and provide the ideal conditions for this cycle to compound poverty and disease for coming generations. As described, a wealth of data links growth faltering to negative health, social and economic outcomes (39); what has also been demonstrated is that the window to most effectively avert these consequences may be quite small. Difficulties in recovering linear growth after infancy and early childhood are well-described, and have led to the identification of "the first 1000 days" spanning conception to 24 months of age as a critical window for intervention (40–42). Further, while the mortality rates associated with acute undernutrition or wasting are higher, the long-term population impacts of chronic undernutrition are relatively greater, and there is a comparative paucity of prevention and treatment programs to tackle it (3,5). Similarly, while mortality due to acute diarrheal disease has decreased in recent years, morbidity has persisted (4,5,43) and is thought to drive enduring deficits in cognitive function and school performance (44–46). A growing body of research also links both enteric disease and stunting in early life to increased risk of chronic diseases in adulthood (43). The rising prevalence of conditions like overweight and obesity, cardiovascular disease, and diabetes, is a formidable challenge to health systems in LMIC and will further compound the resource shortages they face relative to wealthier nations.

The underwhelming impacts of considerable intellectual and material resources that have been allocated to interventions must also be noted. Disappointing results of water, sanitation, nutrition and enteric disease control programs are an obstacle to maximizing health gains and use of valuable financial and human resources in affected populations (47). One important example is lower vaccine efficacy in areas of high undernutrition and enteric disease. Ongoing research is being conducted to promote a better understanding of risk factors for vaccine failure in developing country settings, but it is thought that protein-energy malnutrition and micronutrient deficiency, stunting, infection in early life, and mucosal intestinal damage are all implicated (48–50). Furthermore, recent evaluations of rigorously implemented water, sanitation and hygiene interventions in Sub-Saharan Africa and South Asia have yielded deeply discouraging results demonstrating little to no effect on diarrheal disease incidence or child growth (51).

Given the amount of mortality and disability driven by these conditions, and the failure of programs to effectively interrupt this cycle and achieve gains in child growth, there is an ethical imperative in the public health community to devise novel strategies targeting these pathways.

Novel research & emerging frameworks

In response to these enduring challenges, the public health community has called for a rethinking of the causal pathways driving this cycle and a redoubling of efforts to interrupt it (5). Within this arena there has been a great deal of literature focusing on the structure and function of human intestinal tract, or the site at which these interactions occur. Four distinct layers of defense are maintained in the human gut: a *mechanical* barrier of epithelial cells separating intestinal contents from the inner host; a *mucosal* barrier comprised of antimicrobial epithelial cell secretions; an *immunological* barrier of innate and adaptive immune cells in the mucosa and subepithelia; and an *ecological* barrier comprised of gut microbiota deterring colonization by enteropathogens (30,47). The next two sections will summarize the existing literature and outline the hypothesized role of two distinct but related theories of gut barrier disruption, environmental enteropathy and alterations to intestinal microbiota, in exacerbating the burden of undernutrition and enteropathy.

Environmental enteropathy

During the same era when the cycle of infection and undernutrition was first described, it was also observed that people living in areas of endemic enteric infection exhibited abnormal morphologies of the small intestine (52,53). The condition, coined 'tropical enteropathy,' described flattened intestinal villi and increased inflammation largely in the absence of any perceptible symptoms. In subsequent years, comparison of these observations across geographic and developmental settings confirmed the hypothesis that morphologic gut alterations occur in populations with poor hygienic conditions and high burden of enteric infections, and can be reversible upon relocation to settings with access to hygiene resources, safe water and sanitation infrastructure (47). These physiologic, anatomic and functional changes of the gut are now referred to as *environmental enteropathy* (EE) and are posited to play an important role in the cycle of poverty, undernutrition, compromised immunity, and long-term cognitive and physical impairment in LMIC (54). This hypothesis can be summarized as follows: *infants living in poor hygienic conditions develop EE early in life, experiencing increased gut permeability and possibly malabsorption, which then drive local and systemic immune activation leading to impaired immunological, nutritional and cognitive development* (47,54).

Indeed, there is evidence that EE can help explain interactions between undernutrition and intestinal infections. Chronic stimulation of the immunological barrier by enteropathogens results in over-active innate responses, which in turn may lead to increased permeability of the mechanical barrier and reduced vaccine efficacy (55). Increased epithelial permeability can then results in translocation of microbial products, and xenobiotics, including but not limited to food antigens, from gut contents into general circulation driving systemic inflammation, which is implicated in a range of deleterious health conditions (30,47,54,56). Evidence of the linkage of inflammation and gut permeability to nutritional attainment in LMIC is increasing. Studies in a population of Gambian infants reveal associations between markers of intestinal permeability (IP) and resulting microbial translocation (MT) with significant growth faltering (57–60). While infants exhibited barrier function comparable to UK participants at 2 months of age, IP and MT were found to increase substantially in the Gambian group by 15 months, accompanied by negative trends in linear and ponderal growth. The two conditions were estimated to account for 51-56% of linear growth impairment during this critical window (43,57,60). Analysis of fecal samples of over 500 children in a large, multi-site cohort study (MAL-ED) found that biomarkers of intestinal inflammation were associated with subsequent linear growth faltering, with children in the highest category of inflammation growing an average of 1.1cm less than those in the lowest (61). Further analyses conducted at two of eight MAL-ED sites, Peru and Tanzania, revealed a significant association between the kynurenine-tryptophan ratio, a marker of systemic inflammation, and deficits in both linear growth and immunogenicity to polio vaccine (62). These findings echoed results of a study in Bangladesh which reported significant associations between inflammatory biomarkers of EE, linear growth, and rotavirus and poliovirus vaccine failure (63). Authors estimated nearly half of the change in height-for-age and a quarter of the variation in rotavirus vaccine-induced plasma IgA were explained by selected biomarkers.

These data support the hypothesis that enteric pressure in early life drives changes in gut function, which in turn act on nutritional outcomes and propel the cycle of undernutrition and infection. Nonetheless, there remains doubt as to whether enough is known to intervene; despite mounting evidence and critical evaluation by expert committees, precise case definitions and optimal biomarkers are still lacking, and results of interventions specifically targeting EE with interventions have been inconsistent at best (64,65).

Intestinal microbiota and the gut microbiome.

The human intestinal tract is host to a dense and diverse community of microbes whose genetic make-up is collectively termed the gut microbiome (66). This community is predominantly comprised of bacteria, known as the microbiota, but also includes viruses, fungi and archaea (67). Millions of years of co-evolution have resulted in complex interplay between the structure and function of the human gut and the microbiota residing therein (68). Recently, advances in genetic sequencing techniques have provided the scientific community with tools to study host-microbe relationships in greater detail. The following section will briefly summarize key research pertaining to the putative role of the gut microbial community in enteropathy and growth in early life.

Variation in human gut microbiota. The gut microbial community is comprised of at least 100 trillion microbial cells which can be characterized as either symbionts essential and beneficial to the host, or pathobionts, which are commensal in a healthy host but opportunistically pathogenic (69). Studies have revealed the existence of an identifiable core microbiome, with community structures and functional gene profiles exhibiting congruity across different populations (70–72). However, presence and abundance of specific bacterial taxa vary within and more extensively between individuals (73–75). Understanding and describing how microbial populations vary across age, geography, and phenotypic traits and developing appropriate metrics for identifying 'healthy' or 'high risk' profiles have been a focus of the emerging research.

The human gut is anatomically formed during gestation, where it remains largely devoid of microbiota; during and after birth, acquisition of gut bacteria occurs in stepwise phases according to various early life events (69,76,77). Method of birth, for example, determines whether the gut environment is initially influenced by maternal fecal and vaginal (vaginal delivery) or skin flora (caesarian section) and is associated with changes to the timing and progression of colonization (78–80). A wide body of literature also illustrates dietary factors as key determinants of this ecosystem, with breastfeeding driving dominance of taxa that metabolize human milk oligosaccharides in early life, and subsequent weaning and exposure to complementary foods as key events triggering a further stage of diversification and proliferation of the gut microbial community (69,75,78,81–84). Thereafter, the gut is thought to progress towards an "adult-like" state marked by more stability and resilience, with enduring inter-individual heterogeneities but a markedly reduced variation within the individual (75,76,85–87).

Literature describing variation in microbial profiles may use species-level metrics, such as the presence or relative abundance of particular taxa, as a descriptor of the gut community at different life stages. The ecologic principle of species diversity or richness, which predicts that more diverse communities have increased resilience to interruption or invasion, has also been employed to judge the 'health' of the microbial community. In microbiome research, shifts in relative abundance of different groups or loss of species diversity is frequently referred to as 'dysbiosis,' and is hypothesized to confer deleterious health outcomes (69,85,88–90). However, no clear case definitions or cutoffs for dysbiotic phenotypes are agreed upon in the literature, and the term is often used to describe the presence or absence of microbial populations inconsistently depending on the outcome being measured or the population being studied. Judging the 'health' of the microbial community in early life, during a highly plastic stage marked by sensitivity to external events, presents additional methodological challenges; volatility of bacterial populations during this time does not necessarily denote ill health, but instead may be following a healthy progression to an adult-like state. The stepwise manner of this progression in early life, coupled with observations that dysbiosis of adult microbiota can cause it to resemble the infant microbial community, implies that there exists some optimal rate or pattern of bacterial assembly that occurs in humans as they transition from a sterile fetal gut environment to a stable, 'healthy' mature profile (85). This has led to the development of a 'maturity' metric, which compares the gut community in a given sample to that which would be expected of a healthy child at that same age, as a tool for assessing whether the gut microbial community is implicated in compromised health growth and health of young children in LMIC (66,91). A more complete discussion of the methodology underlying these indicators is provided in the methods section of this document.

Contribution to health. The gut microbial community operates synergistically with the human host to fulfill key metabolic and immunological functions, and is therefore implicated in a wide range of health outcomes. Microflora ferment dietary components that are otherwise indigestible to the human host, producing short-chain fatty acids that can be absorbed by the gut and thereby promoting dietary energy harvest (92–94). Metabolic functions of microbiota also include synthesis of amino acids and vitamins K, B_{12}, and folic acid, and absorption of magnesium, calcium and iron (92,94,95). Transfer of bacteria from over- or under-nourished humans can induce obesity and undernutrition in germ-free mice. That these conditions develop holding diet constant, and can be manipulated and reversed using different microbial populations, further suggests a causal relationship between gut microbiota and differential energy harvest, weight gain, and growth (91,96–99).

Studies in vitro and in animal models have also revealed profound impacts of gut microbiota on the mechanical, immunological and mucosal layers of the intestinal barrier. Substrates of microbial metabolism in the gut contribute to T-lymphocyte differentiation, antimicrobial peptide release from plasma cells during infection, development of gut-associated lymphoid tissues (GALT) and upregulation of tight-junction proteins of the gut epithelia (30,93,100–103). Colonization by microbiota immediately after birth is thought to prime the naïve gut, triggering development, differentiation and maturation of immune cell populations and help to maintain the homeostatic balance between tolerance and inflammation (104–106). Encouraging data from murine models point to the impact of manipulating microbiota to improve immunogenicity to influenza (107), *Salmonella* (108) and rotavirus (109) vaccines. The apparent role of the microbiota in immunomodulation and barrier integrity also provides a plausible linkage to EE, which is marked by disruptions to both. Recent work demonstrated that a particular profile of gut microbiota induced the phenotype of environmental enteropathy, defined as increased intestinal permeability and inflammation, in mice (110).

Evidence that interruption to the gut ecosystem has nutritional and immunological consequences has also been generated in human populations, largely using observational studies. In high-income settings, extensive work has documented differences in microbial populations of individuals with obesity (111–113) and type II diabetes (114,115). Microbial indices are further implicated in an array of inflammatory and autoimmune conditions, with diversity and relative abundance of particular genera differing among patients with inflammatory bowel diseases, including Crohn's disease and ulcerative colitis (116,117), asthma (79), food allergies (118) rheumatoid arthritis (119) and colorectal cancer (120,121). Disruptions to microbiota have also been linked with infectious disease risk, with the most convincing causal evidence illustrated by the case of *Clostridium difficile.* Sustained antibiotic use in hospital settings profoundly compromises the microbiota, allowing colonization by *C. difficile* (122). The ensuing symptoms, which range from diarrhea to fatal forms of colitis, can be treated with fetal microbiota transplantation to restore the gut microbial ecosystem (123). In vitro and murine studies have also recently demonstrated changes to intestinal microbiota as factors in enabling infection, colonization and pathogenesis of diseases of *Giardia* and *Campylobacter spp.,* enteropathogens of significant public health relevance in LMIC (124,125). These particular infections have been cited as illustrative examples of the EE hypothesis, due to their contribution to diarrhea as well as asymptomatic enteropathy and their associations with long-term developmental sequelae. Characterization of microbial risk factors or consequences of infection with these enteropathogens will be important to understanding the role of the microbiota in driving this cycle.

These data have largely been generated in ‘developed’ contexts providing insights for populations living in environments with a marked lack of exposure to pathogenic and nonpathogenic microbes early in life (126). Equivalent work on relationships between the microbiome, immunomodulation, and disease risk in areas further upstream in the epidemiologic transition, those in which the cycle of enteropathogens pressure and undernutrition is most dramatic, is comparatively lacking.

Microbiota and child growth and immunity in LMIC. Studies among young children in LMIC do suggest that significant differences exist in the microbiota according to nutritional profiles. This evidence is strongest when severe, acute undernutrition, or low weight-for-length, is the key exposure or outcome. Reductions in global diversity and the overall number of OTU, and differences in presence and abundances of numerous taxa, have been observed to correlate with weight-for-length profiles in Bangladesh, Malawi, Niger and Senegal (66,97,127,128). Children with severe and moderate acute malnutrition were also found to have significantly reduced microbial maturity; while this improved during nutritional therapy, gains were transient and declined within 4 months of treatment cessation. Taken together, these studies demonstrate that children with acute malnutrition appear to have distinct populations and rates of microbial progression in early childhood in a manner that may endure even beyond treatment. Further, the induction of wasting in mice upon fecal transplantation from study subjects in Malawi and Bangladesh (91,97) convincingly demonstrates that these microbial communities impact weight gain. This has led to interest in the development of microbiota-directed therapeutic foods and probiotic interventions for the resolution of acute undernutrition (128,129); however, the studies detailed remain limited in number and sample size and no experimental trials have yet been conducted. Nonetheless, they serve to illustrate the biological plausibility that gut microbiota are implicated in the cycle of undernutrition in LMIC.

Fewer studies have considered the relationship between microbiota and chronic undernutrition, which drives a greater proportion of long-term disability worldwide. Community-level, taxonomic and functional alterations to the microbiota have been observed among children with linear growth shortfalls in India, Malawi and Bangladesh. However, none interrogate the pathway of linear growth failure in a specific manner; instead, all enroll children based on a composite malnutrition score inclusive of other phenotypes like low birth weight or ponderal growth failure, or conduct retrospective analyses of children initially enrolled in studies of wasting (130–132). Studies designed to measure this relationship in a more specific manner, and among more representative samples of young children, are needed to elucidate the potential role of the gut microbial community in driving chronic undernutrition in LMIC.

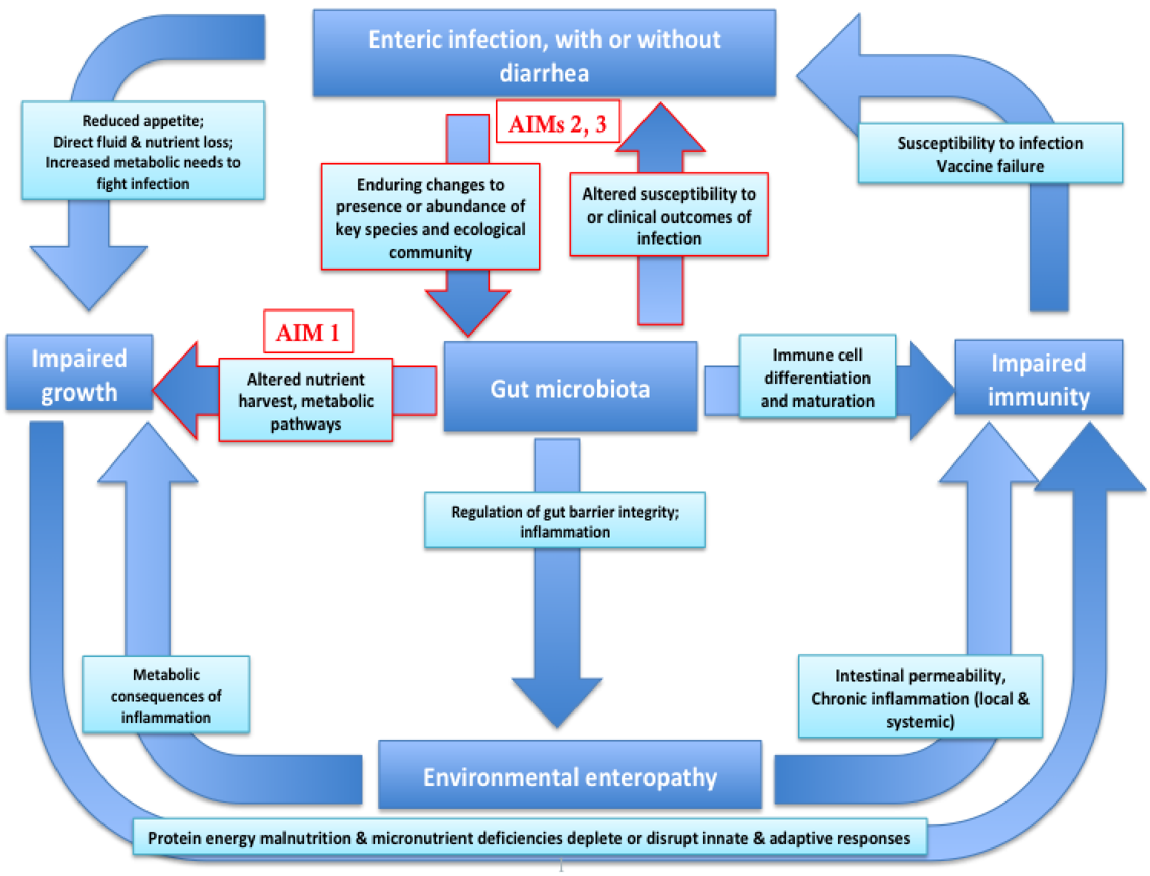
Studies assessing gut microbial impacts on enteric immunity and disease in this demographic have also emerged in the literature. Recent work suggests that diarrhea can have lasting impacts on the bacterial communities in the gut, citing differences in taxonomic composition, diversity and maturity during and after illness (66,133–135) in young children in LMIC. Few human studies have been conducted to replicate findings that microbiota impact the ability to launch effective immune responses, though this has been suggested by the observation of heightened response to oral *Salmonella typhi* (Ty21a) vaccine (136) as well as systemic *E. coli* and *Salmonella infections (137).* Investigators have further explored this question by measuring impacts of probiotics on immunogenicity and vaccine efficacy, with disparate and often disappointing results (138). This may be due to the lack of consistency of the type, dose and duration of pre- and probiotic treatment; the age, ethnicity, and socioeconomic and geographic profiles of participants; and the kinds of vaccines studied. Evidence assessing the potential interplay between microbiota and features of EE such as asymptomatic infection, inflammation and intestinal permeability are even more scarce. To our knowledge, there is no published work specifically assessed the role of asymptomatic enteric infections on the gut microbial community, and only one recent study conducted in Malawian children found significant differences in presence and abundance of particular taxa among children with severe EE (139).

While this evolving body of work provides a compelling case for the possible role of the microbiota in driving the interactions between undernutrition and enteric infection and disease, there exists a clear need to elucidate potential mechanisms further and build an evidence base that is inclusive of broader populations of children in LMIC. Recently, experts in the field of childhood undernutrition have called for the specific interrogation of pathways through which infection may alter the microbial environment, and a more comprehensive description of associations between gut microbial environment and features of EE, as research priorities for achieving global targets in the reduction of childhood stunting worldwide (140). The following section distills these theories into a theoretical framework and outlines the specific aims of this dissertation.

Theoretical framework & specific aims

Figure 1 depicts the described relationships between enteric infection and illness, undernutrition, and compromised immunity and illustrates ways in which they may be driven by microbiota in the gut and EE. The current dissertation was conceived to assess these relationships in the Santa Clara Cohort, a birth cohort of 303 children enrolled at one site (Peru) of the multi-country cohort study entitled 'The Interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development (MAL-ED).' The first component of this work aims to describe relationships between anthropometric and microbial indices in the first two years of life, the most critical postnatal window for growth acquisition and nutritional intervention. Subsequent analyses were designed to detect possible impacts of diarrheal disease and asymptomatic infections on the gut microbial community, which has been highlighted as an important research objective in the effort to elucidate how intestinal infections and subsequent illness illicit long-term disabilities via growth and immunological impairments throughout life.

Figure 2: Theoretical framework illustrating possible role of the gut microbiota in driving the cycle between enteric infections and undernutrition in LMIC



Theories were summarized using data from the studies summarized in the previous section with citations listed at the end of this chapter. Pathways investigated in the specific aims of this dissertation are shown in red.

Specific aims. Specific aims of this research were designed to interrogate relationships between the gut microbial community, metrics of growth acquisition, and symptomatic and asymptomatic enteric infections in early life (pathways shown in red above). We sought to first replicate findings described elsewhere that children with different growth profiles also have detectable differences in their gut microbial community (Aim 1). We then measured whether alterations in the gut community are associated with increased incidence of all-cause diarrhea, and in turn whether diarrhea has lasting impacts on the gut community (Aim 2). Finally, we sought to link these questions to the theory of EE, by partitioning analyses into symptomatic and asymptomatic carriage of a single enteropathogen and chronicling whether and how these exposures may impact microbial species composition in the gut (Aim 3). *Campylobacter spp* infections were selected for this analysis due to their high prevalence in this population and their observed impacts on child growth and other long-term disabilities, both in this cohort and elsewhere. The underlying hypothesis of these analyses was that gut microbial indices would both reflect ponderal and linear growth disparities among children in this community, and be detectably altered by frequency or intensity of both symptomatic and asymptomatic enteric insults. Specific aims are listed below:

Aim 1: *Detect and describe associations between gut microbial metrics at 6, 12, 18 and 24m and growth acquisition in the Santa Clara cohort*

> **1a):** *Assess whether gut microbial maturity (MAZ) is indicative of ponderal (WLZ) and linear (LAZ) growth deficits from birth to two years of age*
>
> **1b):** *Assess whether measures of gut microbial diversity and richness differ among children with linear growth faltering from birth to two years of age*

Aim 2: *Detect and describe bi-directional associations between diarrheal disease and microbial diversity and richness, and assess whether associations endure over time among children age 0-24m in the Santa Clara cohort*

2a) *Measure impacts of diarrheal frequency, duration and severity on subsequent metrics of microbial diversity and richness in the infant gut*

2b) *Measure associations between measures of gut microbial and richness and subsequent diarrheal incidence from birth to two years of age*

2c) *Measure whether and how detected associations differ by stunting status, weaning status, and time*

Aim 3: *Evaluate associations between Campylobacter infection, community and species-level of gut microbial metrics, and child growth among children 0-24m of age in the Santa Clara Cohort*

3a) *Assess impacts of symptomatic and asymptomatic Campylobacter infections on measures of gut microbial diversity and richness*

3b) *Identify whether particular gut microbial members are associated with Campylobacter infections among children in the Santa Clara cohort, and assess evidence of their relationship to linear growth among children from birth to 24m of age*

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Chapter 3: Study Context & Methodology

The current study is nested within the MAL-ED Study, a collaboration between the National Institute of Health, the Fogarty International Center, the Bill & Melinda Gates Foundation, and a broad network of biomedical research institutions and eminent investigators at the forefront of research in child health, nutrition, environmental and specifically enteric disease, and cognitive development (1–11). The MAL-ED Study is a longitudinal birth cohort of children from 0-60 months of age in eight countries who continue to suffer the consequences of the cycle between poverty, undernutrition, enteropathy, and compromised immunity. Communities in each of the eight participating countries were selected and followed according to a unified protocol to provide a more complete description of the inter-relationships between enteropathy, growth, immunity, and cognitive development and inform intervention design for children living in poverty. Detailed descriptions of MAL-ED protocol and methods have been published and are referenced throughout the subsequent chapters (1–4,8,10,12,13); however, the following section provides a brief description of the data collection and diagnostic methodology employed in the parent study.

Sample selection & enrollment

Fieldworkers performed a community census to enumerate populations of women of reproductive age. Catchment areas were then identified with an aim of recruiting at least 200 children per site. Healthy infants were enrolled within 17 days of birth and followed longitudinally until 24 months between November 2009 and February 2012. All sites received ethical approval and informed consent. Inclusion and exclusion criteria for the MAL-ED Study are listed in Box 1.

Box 4: Criteria for enrollment in the MAL-ED Study across eight sites (8)

Inclusion Criteria

- **Healthy infant enrolled within 17 days of birth**
- **Caregiver report that they had no plans to move out of catchment area for at least 6 months following enrollment**
- **Willingness to be visited in the home twice weekly**

Exclusion Criteria

- **Reported plans to move out of catchment area for a period of >30 days within 6 months of proposed enrollment date**
- **Maternal age < 16 years**
- **Sibling enrolled in MAL-ED study**
- **Child was not a singleton**
- **Indications of serious disease:**
    - **Hospitalization for atypical birth**
    - **Severe or chronic condition diagnosed by medical doctor**
    - **Enteropathies diagnosed by medical doctor**
- **Guardian did not provide consent**
- **Weight at birth or enrollment <1500g**

The current work is nested within one site, MAL-ED Peru, in Santa Clara de Nanay.

MAL-ED Peru: Santa Clara de Nanay

Peru is an upper middle-income country of 30.4 million people in South America with a vastly diverse geography and ethnic composition. While it is among the fastest growing economies in Latin America, with a gross domestic product of over $202 billion (14), populations living in urban slums and remote coastal, mountainous or Amazonian regions are persistently characterized by poor socioeconomic, health and nutritional indices (14). The Department of Loreto, located in the lowlands of the Amazonian jungle, is one such region.

Loreto is geographically Peru's largest region, with international borders with Ecuador, Colombia, and Brazil. According to a 2012 national census by the National Institute of Statistics and Informatics, Loreto is home to 1.1 million inhabitants, nearly half of whom (457,900) are based in the departmental capital of Iquitos.

This Amazonian region experiences consistent rain throughout the year, with an annual peak in January during which flooding is common. The average annual precipitation is 3.4 meters and the average temperature is 25.8° C/78.4° F. Seasonal and annual variations in precipitation and climate are important factors determining the economic and nutritional profiles of these communities, who often rely on fishing, logging and farming for subsistence and livelihood. Loreto region consistently demonstrates some of the poorest health and economic indices in the country (11). Access to water, sanitation, and livable wages is markedly lower than elsewhere in Peru, and the burden of infectious disease remains high. Infant and child mortality are both considerably higher than the national average (see Table 1).

Table 2: Health indicators comparing Peru, Loreto region, and the MAL-ED Cohort, adapted from (11)

Indicator	Peru	Loreto	MAL-ED Cohort
Access to health services			
Clean water (% of households)	72.5	23.8	NA
Improved sanitation (% of households)	58.4	27.1	20.2
Receive all vaccinations (% of children)	71.0	53.9	NA
Delivering in facility (% of women)	85.1	53.1	81.3
Receive postnatal care (% of women)	69.9	40.9	NA
Health outcomes			
Life expectancy (years)	72.5	69.9	NA
Children born underweight (% born under 2.5 kg)	6.5	9.1	10.6
Proportion of U5 stunted (%)	19.5	31.0	46.3
Proportion of U5 wasted (%)	0.4	1.9	0.2
Proportion of U5 underweight (%)	0.5	8.9	5.6
Proportion of U5 with reported diarrhea in past 7 days	13.9	30.4	35.4
Under 5 Mortality Rate (per 1000 live births)	21.0	60.6	NA

The MAL-ED team in Peru is based out of Santa Clara de Nanay, a research facility situated approximately 15km southeast of Iquitos. This site covers participants from three rural communities, Santa Clara, Santo Tomas and La Union, located along the Nanay River. The approximately 5000 families in these communities are estimated to be demographically representative of the population in Loreto region (11). A collaboration between the Johns Hopkins Bloomberg School of Public Health, the Asociation Benefica PRISMA and the United States Naval Medical Research Unit No. 6 has been operating and conducting research from this site for over a dozen years. A total of 303 children were enrolled for inclusion in MAL-ED at the Santa Clara de Nanay fieldsite, with an expected high loss to follow-up due to frequent seasonal displacement and an aim to retain a minimum of 200 children in the study.

Data collection

Measurement of demographic and nutritional covariates. At enrollment, date of birth, sex, birth weight, length, weight, head circumference, and details on breastfeeding initiation were recorded. Ongoing information on diet and breastfeeding was collected during surveillance visits monthly in the first year of life and quarterly in the second and is described in detail elsewhere (10). A complete list of dietary exposure variables and their collection source is shown in Box 2.

Box 5: Dietary exposures collected in MAL-ED (10)

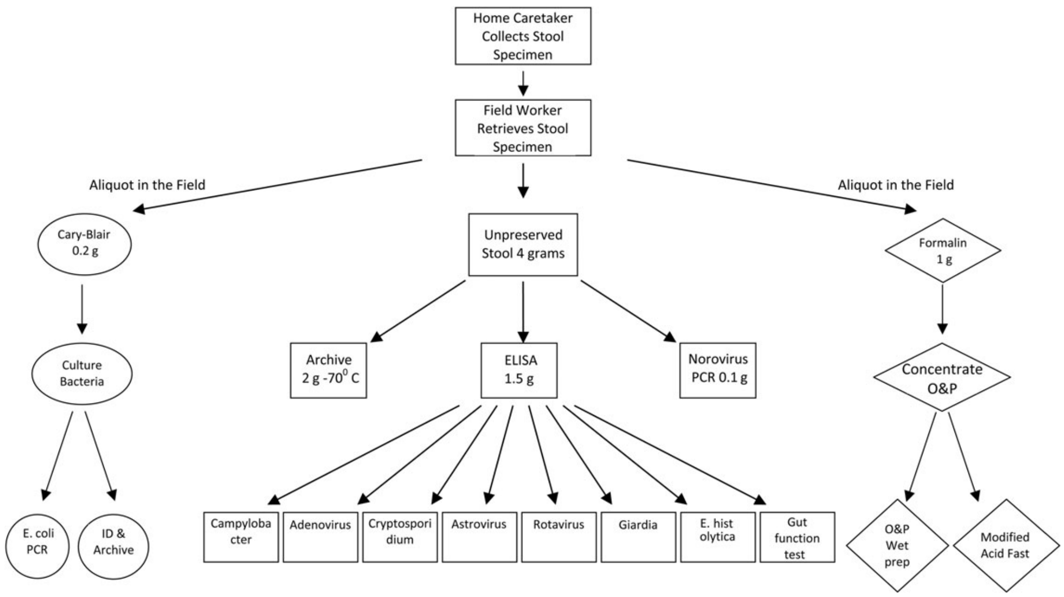
Dietary Exposure	Source of Data
Breastfeeding	
Prelacteal feeding	Enrollment interview
Timing of initiation	Enrollment interview
Exclusivity (exclusive, partial)	Biweekly and monthly interviews
Age at complete weaning	Biweekly and monthly interviews
Infant feeding practices	
Age at introduction of non–breast milk liquids, semisolids, and solids	Biweekly and monthly interviews
Age at regular consumption of semisolids and solids	Biweekly and monthly interviews
Dietary intake	
Energy, macronutrient, and micronutrients	Monthly 24-h recalls
Nutrient adequacy ratios	Monthly 24-h recalls
Probability of intake inadequacy	Monthly 24-h recalls
Overall dietary quality	
WHO indicators (frequency, diversity, minimal acceptable diet, intake of iron-rich foods, intake of vitamin A–containing foods) (6–24 mo)	Biweekly and monthly interviews Monthly 24-h recalls
Mean adequacy ratios	Monthly 24-h recalls
Biomarkers of dietary intake	
Plasma retinol	7, 15, and 24 mo
Plasma zinc	7, 15, and 24 mo
Plasma ferritin	7, 15, and 24 mo

Abbreviation: WHO, World Health Organization.

Dietary recall questionnaires started at 9 months of age

Stool collection and microbiology. Routine stool collection was conducted monthly, with additional samples collected during diarrheal episodes occurring in between planned routine visits. Diarrhea was defined as ≥3 loose stools in a 24-hour period and separated by at least two diarrhea-free days. Children experiencing moderate to severe diarrhea were referred to local health services. Caregivers were enlisted to collect fresh stool and specimens were collected and transported to the laboratory for processing monthly on the day of their birthday or upon occurrence of diarrhea. A depiction of enteropathogens assays is shown in Figure 1, with detailed description of bacterial culture methods, immunoassays for protozoa and viruses, and microscopy harmonized across all eight sites described elsewhere (12).

Figure 1: Microbiologic methods for enteropathogen identification in MAL-ED (12)



Surveillance. A surveillance system was designed for all MAL-ED sites to quantify common exposures and disease states that may be associated with growth and development in early life (13). Active surveillance was conducted to provide daily illness and treatment history for children and identify diarrheal episodes for specimen collection. Illness and treatment history was collected twice-weekly by fieldstaff. Visits were made every 3-4 days to ask mothers or caregivers whether children experienced symptoms or were treated with any medications featured in Box 3. Answers to these questions were then used to assign illness categories based on pre-specified case definitions for common childhood illness, such as acute lower respiratory infections and diarrhea. In cases of missed surveillance visits, recall was ascertained for no more than 7 days prior. All data collection materials were translated into Spanish, and verbal questionnaires were also conducted by fieldstaff from the region in their native language.

Box 6: Questions featured in illness and treatment history questionnaire, administered twice-weekly in MAL-ED (13)

Questions	Responses
Section A. Overall health	
Was the child sick?	No; Yes
What was the child's activity level?	Normal; Sleepy; Difficult to awaken
How was the child's appetite?	Normal or more than normal; Less than normal
Did the child vomit?	No; Yes
Did the child have ear pain/ pulling?	No; Yes
Section B. Enteric infection	
Did the child have diarrhea?	No; Yes
How many loose stools did the child pass? (loose stools take the shape of their container)	0–99
Was there any blood in the child's stool?	No; Yes
Was the child dehydrated?	No Some: irritable, thirsty, delay in skin pinch, sunken eyes Severe: symptoms more severe, with lethargy, listlessness
Section C. Other illnesses	
Did the child have an illness with a cough?	No; Yes
Was the child short of breath?	No; Yes
Has the child been ill with a fever?	No; Yes
Section D. Field staff measurements	
Field staff observe the child's chest for indrawing.	No; Yes
Field staff members take the child's respiratory rate, twice.	Breaths per minute
Field staff members take the axillary temperature if fever is reported.	Degrees Celsius
Section E. Treatments	
Did the child take oral or injected antibiotics?	No; Yes
What kind of antibiotics did the child take?	Penicillin; cephalosporin; sulfonamide; macrolide; tetracycline; fluoroquinolone; metronidazole; other; or unknown
Was child given oral rehydration salts, a prepackaged oral rehydration salt solution, or a government-recommended homemade fluid?	No; Yes

Data collected in sections A, B, C, and E are based on mother or caregiver report.

Quality control

A technical subcommittee within MAL-ED was established to produce standard operating procedures, checklists and questionnaires and provide guidelines and uphold minimum quality standards across all MAL-ED sites (13). Field supervisors reviewed all forms prior to entry and conducted surveillance on 10% of households monthly for quality control purposes. The percent agreement at the Peru site was above 90% for classification of any illness, diarrhea, fever and antibiotic use. A central Data Coordinating Center was also assembled for additional quality control screening for all sites (13).

Ethical considerations

All participants provided written informed consent as a prerequisite to inclusion in the study. Consent forms were read to parents and/or guardians. A copy of a consent form was left in the family's possession. This study received ethical approval from the Johns Hopkins Institutional Review Board as well as from the partner organization, Asociation Benefica PRISMA, in Iquitos, Peru.

The Peru Microbiota Collaboration

The Peru Microbiota Collaboration was launched in 2015 with the Gordon Laboratory at Washington University in St Louis, one of the foremost institutions leading innovations in genome science and technology in the world, to link the epidemiologic data generated by The Iquitos Satellite Laboratory (IQTLAB), the institution responsible for research at the Peru site of the MAL-ED Consortium, to indicators of gut bacterial composition and functioning. Led by Dr. Jeffrey Gordon, a pioneer in establishing the role of the gut microbiota in human health and promoting translational research for the development of clinical interventions, this institution is a leader in publishing novel methodologies in microbial and metagenomic analyses and linking derived indices to issues of public health importance (15–24). In August 2017, the Gordon Laboratory completed bacterial and genomic analysis of over 1000 fecal samples from 303 children in the Peru cohort, comprising the largest longitudinal database of gut microbial indices from infants living in LMIC to our knowledge.

Enrollment/inclusion

All 303 children were eligible for inclusion in the study, irrespective of subsequent loss to follow up. Two inclusion processes were executed: the first, to select a sub-cohort of children exhibiting 'healthy' growth to serve as a reference population for the generation of microbial maturity metrics; and the second, to select from stools contributed by the full population within which to conduct analyses for aims 1-3 of this work.

Selection of the subsample of children with unperturbed growth was conducted as follows: children were eligible for inclusion if their weight-for-length and length-for-age Z-scores, denoting wasting and stunting histories respectively, were never detected to be below -1.5 standard deviations below 0 between birth and their 2nd birthday. Twenty-two children met these criteria. Monthly routine stools for these participants were then sent to the Gordon Laboratory for to comprise a reference population for subsequent comparison of age-discriminatory taxa (described below).

All children in the cohort (n=303) were eligible for inclusion in the study. For each child, we selected the routine stool sample available at 6, 12, 18 and 24m of age. Where this sample was missing, we selected the surveillance stool collected 1m preceding or following the optimal age, with preference for whichever was collected the shortest amount of time from their 6, 12, 18 or 24th month dates. This produced a sample of 271 children at baseline, and 200 children at endline.

Processing of stool samples for microbial analysis

DNA was isolated from aliquots of pulverized, frozen fecal samples. Fecal samples were homogenized by bead beating for 4 minutes (MiniBeadbeater-96™, Biospec Products; Bartlesville, Oklahoma USA) in a mixture of 250 µL of 0.1 mm-diameter zirconia/silica beads (and a 3.97 mm-diameter steel ball for a subset of samples), 710 µL of 500:210 2X buffer A (200 mM NaCl, 200 mM Tris, 20 mM EDTA):20%SDS, and 500 µL of 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. Samples were then centrifuged at 3,220 x g for 4 min at room temperature. 350 uL of aqueous phase was transferred to a new tube containing 500 uL 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. The tubes were inverted 10 times to mix and then centrifuged at 3220g X 4 min. 250 uL of aqueous phase was transferred to a 96-well plate. DNA was purified from a 100 uL aliquot of each 250 uL crude DNA sample by mixing 100 uL of crude DNA with 400 uL of a 675:45 mix of Qiagen® buffer PM:3M NaOAc, pH5.5 and then passing the mixture through a Qiagen® QIAquick96 plate by centrifugation at 3220g X at least 4 min. Wells were washed twice by passing 900 uL Qiagen® buffer PE through the plate by centrifugation at 3220g X at least 2 min. Quant-iT dsDNA Broad-Range Assay Kits (Invitrogen; Carlsbad, California, USA) was used to quantify the pure DNA.

For each DNA sample, the V4 regions of bacterial 16S rRNA (V4-16S rRNA) genes present were amplified using previously published primers (25) and the following PCR conditions: an initial denaturation step of 2 min at 94°C, followed by 26 cycles of 15 sec at 94°C, 30 sec at 50°C, and 30 sec at 68°C, followed by a final step at 68°C for 2 min. Amplicons were sequenced using an Illumina MiSeq instrument. The resulting 250-nucleotide paired-end reads were trimmed to 200 bases and merged with Flash, and

QIIME (v 1.9.0) (26) was used to demultiplex the reads and cluster them into operational taxonomic units (OTU; clusters sharing ≥97% sequence identity). OTU that failed alignment by PyNast (27) were removed from the dataset. Taxonomy was assigned to each OTU using the Ribosomal Database Project (RDP) classifier version 2.4 (28) and the 2013 Greengenes reference database (29). The resulting OTU table contained 32,300±16,453 (mean±sd) reads per sample. Subsequently, OTU that did not have relative abundances of ≥1% in at least one sample or ≥0.1% of the reads in at least two samples were removed from the data. The data were then rarefied to 11,000 reads per sample, and Shannon's diversity index, the total phylogenetic diversity, and the observed number of OTU for each community were calculated as measures of microbial richness and diversity. Samples that did not have at least 11,000 reads were removed from the data.

Generation of outcomes of bacterial diversity & maturity

Bacterial diversity and richness. Alpha diversity metrics are commonly employed in community ecology to describe the variation in species composition within a given sample within a given community. Diversity metrics account for richness, the number of overall species present in a sample, and the abundance of each. Four metrics of species richness and diversity were utilized in this study, described below.

Shannon diversity index (SDI). The Shannon Diversity Index (SDI) accounts for the number of species (richness) and their distribution (evenness) to capture diversity within samples. SDI describes the amount of information in a given sample using the sum and proportional abundance of each particular OTU, with equal sensitivity to rare and abundant species (30). Higher SDI values denote greater species richness and evenness;

a maximum value for this metric is therefore reached when all species within a given sample are perfectly evenly spread. The formula for this metric is shown below, where p_i is the proportion of species *i* relative to all species in a given sample.

$$\boxed{H' = -\sum_{i=1}^{R} p_i \ln p_i}$$

CHAO1 Estimator. The CHAO1 estimator of species richness is a nonparametric method that calculates the total number of species in a given sample as a function of the number of species observed, and the probability that they are observed more than once in the same sample (31,32). This method is adapted from the mark-recapture-release statistics employed for counting animal populations (33,34), and assumes that the probability of observing the same species more than once is greater in a sample with lower diversity. The formula for the CHAO1 estimator is shown below, *where* S_{obs} is the total number of species observed*;* n_1 is the number of species observed once*;* n_2 is the number of species observed twice (re-captured):

$$\boxed{S_{\text{Chao1}} = S_{\text{obs}} + \frac{n_1^2}{2n_2}}$$

Faith's Phylogenetic Diversity (PD). The PD metric estimates richness in an ecological community by calculating the sum of the branch lengths of phylogenies within a given sample (35). This measure does not account for abundance of species, but incorporates the degree of divergence between species in its assessment of richness: two taxa closely related will together contribute less to the overall metric, by virtue of their shared phylogenetic material, than two taxa that are highly divergent.

Simpson's diversity. Simpson's diversity index (36) estimates the probability that randomly selected species within a sample, selected with replacement, will be different. This metric is calculated as the sum of the proportional abundances of all OTU within a given sample, and is bound by 0 and 1 as it represents a probability. The Simpson index is weighted towards more dominant species, which are more likely to be sampled at random.

Microbial maturity. Maturity measures have specifically been developed to describe the possible differences in development and maturation of the infant gut microbiome that may be implicated in differential growth and nutritional uptake during a critical postnatal period (25). Identification of microbial taxa that characterize children exhibiting healthy growth at different chronological ages provides a template against which to compare other individuals (25). Metrics derived from age-discriminatory characteristics of the gut environment are referred to as indicators of microbial maturity.

Microbiota-for-age Z scores (MAZ). To generate microbial maturity metrics, the relative abundance of each OTU was regressed against the age of the child using a Random Forests Machine Learning Algorithm (RF). The RF technique is a statistical procedure identifying distinct groups of a population whose members share a common association with the predictor of interest; in this case, the procedure identifies the predominant groups of taxa associated with each age bracket (month of life). Evaluation of high-throughput sequencing techniques for characterization of the human microbiome cites RF as among the most accurate classification methods for microbiome data, judged on ability to correctly classify groups and produce minimal levels of uncertainty in modeling their association with descriptors of interest (37).

Age-discriminatory taxa derived using RF were ranked at each age group in order of their contribution to model accuracy. Species were ordered based on the degree to which their rank permutation increased uncertainty in the predictive accuracy of the model, as measured by the mean-squared error. This procedure was first conducted among 50 healthy infants in Bangladesh, and the derived model was applied to other healthy populations to determine its ability to predict age-discriminatory taxa in different populations. Application to two other groups within Bangladesh (13 healthy singletons and 25 healthy twins and triplets) supported the consistency of the 'signature' of OTU at each age (25). The model was also found to generalize to a cohort of 47 twins and triplets exhibiting healthy growth in Malawi (25), demonstrating predictive accuracy across geographic locales and environmental pressures. Further cross-validation has recently been conducted with an aggregated sample of 27 children from Peru, Brazil, India and South Africa. These data support the use of this model to accurately predict the presence of key microbial taxa in the healthy infant gut across populations; most encouragingly, when the Bangladesh model was applied to a dataset of 507 samples from 22 children with healthy growth included in our analysis, it demonstrated strong predictive accuracy (r^2=0.76; Ahmed, Huq & Subramanian, unpublished).

The 'microbiota age' of a fecal sample, therefore, is the age corresponding to the signature of taxa in that sample. Among healthy children, this corresponds to the chronological age of the child; but among children exhibiting different growth trajectories, the microbiota age may not be aligned with what is expected for a healthy child of the same chronologic age. The microbial maturity metric is then calculated as a standardized Z-score as follows (25):

$$\textit{Microbiota-for-age Z-score} = \frac{\text{(microbiota age- median microbiota age of healthy child of same chronological age)}}{\text{(standard deviation of microbiota age of healthy children of that chronological age)}}$$

Analytic approach

The advantage of the study design of MAL-ED and other longitudinal cohorts is the ability to measure both risk factors and outcomes as they change over time, providing more valid estimates of exposures and allowing temporality of cause and effect to be monitored. The current study employs both cross-sectional and longitudinal statistical methods to characterize relationships between predictors and outcomes throughout the first two years of life.

One distinctive feature of longitudinal data is the clustering of observations within each individual. Repeat measurements over time violate the critical assumption of independence between observations; it is likely that metrics measured within the same individual will be more correlated with one another than with observations from other individuals. Thus, there is a vector of responses for each individual, with the adapted assumption that each response vector is independent; in other words, the vector of microbial outcomes measured within one child does not depend on those measured within another. A single value is used to represent exposures that do not vary over time, like gender or baseline characteristics, and a vector of covariates is generated for time-varying exposures like infection status. The covariance of responses and exposures within individuals must be statistically accounted for in order to avoid incorrect inferences based on measures of precision calculated under the assumption of independence.

The current study employs a generalized estimating equations (GEE) approach (38,39) as a statistical strategy to adjust for within-child clustering. This approach models the mean response across the population while accounting for clustering of responses within individuals over time. An important strength of this approach is that it requires few assumptions about the characteristics of the covariance of correlated observations, allowing for valid inference without making distributional assumptions. This is a strong justification for its use with our outcome data; due to the unstable nature of microbial measures in early life when the gut is very plastic (40) and the relatively novel metrics being used, fewer assumptions about how measurements relate to one another over time may inform more valid models. In addition, while greatly detailed characterization of microbial markers over time has been conducted in the form of within-person panel studies, population-level epidemiologic studies remain absent. Thus, we employ GEE to estimate population-averaged trends in microbial indices according to enteric exposures among children over the first two years of life.

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Chapter 4: Childhood undernutrition and patterns of microbial maturity, diversity and richness in the developing infant gut: results of a 24-month longitudinal cohort in Peru

ABSTRACT.

Background. The global burden of undernutrition underlies nearly half of all under-five mortality and drives considerable loss of human potential among children living in poverty. Interventions to promote growth have been disappointing, calling for novel approaches to understand and target pathways implicated in growth faltering. Recent evidence suggests there may be casual relationships between the gut microbiota and child growth, but representative studies are needed to provide a more complete description of these relationships at the population level. We describe associations between gut microbial maturity, diversity and linear and ponderal growth acquisition in a birth cohort of 271 children aged 0-24m in Peru.

Methods. Routine fecal samples collected at 6, 12, 18 and 24m of age (n=928) were used to identify gut bacterial taxa with polymerase chain reaction (PCR) primers detecting 16S ribosomal RNA, and resulting data was used to generate metrics of gut microbial maturity (microbiota-for-age Z-score; MAZ), diversity and richness (Shannon, Simpson, CHAO1 and phylogenetic diversity). Monthly anthropometric measurements were used to calculate length-for-age (LAZ) and weight-for-length (WLZ) Z-scores. Longitudinal associations between gut microbial metrics and patterns of linear and ponderal growth were assessed using a generalized estimating equations approach to adjust for within-child clustering and produce population-averaged effect estimates. We conducted sub-analyses restricted to children born stunted and those severely stunted. Analyses were adjusted for diarrhea, infection, breastfeeding, dietary diversity and antibiotic exposure.

Results. Two-thirds of children (67%) were stunted, while only 9% of children experienced wasting. Children with lower LAZ had higher WLZ on average (β=-0.24, $p<0.001$). Measures of gut microbial diversity and richness increased significantly with age and weaning, and were suppressed by breastmilk exposure. We detected a suggestive relationship between microbial maturity and WLZ one month thereafter (β=0.03, p=0.071) after adjustment for breastfeeding. We did not observe evidence of associations between microbial maturity, diversity or richness with LAZ in the full cohort. However, MAZ was positively associated with LAZ among the children who were wasted (β=0.55, p=0.004), and LAZ at birth was significantly associated with MAZ score throughout follow-up (β=0.10, p=0.012). Children born stunted had significantly lower gut microbial diversity and richness (Shannonβ=-0.19, CHAO1 =-9.75; p-values <0.05) from birth to two years of age, and there was significant evidence that faltering of diversity acquisition trajectories among these children was exacerbated by cessation of breastfeeding before 24m of age.

Conclusions. We present data describing trends in microbial maturity, diversity and richness, and describe their associations with child growth in one of the largest cohorts to profile these relationships to date. Findings support the hypothesis that gut microbial maturity is related to weight gain among young infants in lower and middle-income countries. We detect deficits in gut microbial diversity and richness among children born stunted, and demonstrate evidence that these deficits endure two years after birth and are

greater among children who are weaned before their 2nd birthday. This provides impetus for programs to emphasize messaging around continued breastfeeding as an important component for nutritional and intestinal health.

INTRODUCTION.

The global burden of undernutrition underlies nearly half of all under-five mortality (1). Stunting, a condition denoting linear growth failure or low length-for-age, continues to affect over 160 million children worldwide (1,2). In contrast with ponderal growth deficits resulting from acute malnutrition, stunting results from chronic nutritional insufficiency, with intergenerational consequences compounding long-term health and socioeconomic inequities worldwide (3). Furthermore, while acute undernutrition carries with it a higher relative risk of mortality, a far greater proportion of children suffer from chronic undernutrition and its consequences (1). Despite the clear evidence of its detriments to the survival and development of vulnerable children, understanding of the full spectrum of mechanisms driving linear growth failure remains incomplete and interventions to prevent stunting have largely failed (4). Strategies to address this gap range from expanding equitable access and uptake of evidence-based programs, to investment in novel genomic and basic science research to elucidate precise pathways that may inform innovative intervention targets.

Given the growing body of evidence implicating intestinal microbes in human health and nutrition, there is increasing interest in whether gut microbial composition, or the composite functional metabolic profile of the microbial community may help to understand linear growth failure in populations living in poverty. The intestinal microbial community has been shown to regulate metabolism and digestion by fermenting and harvesting dietary components, and transfer of bacteria from over- or under-nourished humans can induce obesity and undernutrition in germ-free mice (5–9). While human studies demonstrating the importance of gut flora in nutritional conditions such as obesity and type II diabetes (10–14) are more abundant, evidence in lower- and middle- income countries (LMIC) implicating intestinal flora in undernutrition are only now emerging.

Studies conducted in Malawi and Bangladesh comparing healthy children to those with acute malnutrition, or low weight-for-length, have suggested that there are differences in presence and abundance of specific phyla and reductions in overall bacterial diversity among children with severe acute malnutrition (SAM) (5,15–17). Three studies to our knowledge have considered the relationship between linear growth and microbial indices. One study conducted in 20 rural Indian children classified undernutrition by constructing a composite score representing both linear and ponderal shortfalls, and found community-level, taxonomic and functional differences were associated with the overall condition of malnutrition in this group of children examined at a single point in time (18). Specific indicators of linear growth deficits were not investigated. A second study conducted a retrospective case-control analysis using data from Malawi and Bangladesh children whose microbiota were initially assessed for associations with SAM (19). In a comparison of cases and controls from both countries, investigators noted reduced diversity and increased abundance of *Acidaminococcus* among children with lower height-for-age. Finally, a third recent study compared 10 cases of children with low birth weight and persistent stunting and documented episode(s) of diarrhea in the first 24 months of life to healthy controls, who had a normal birth weight, were not stunted nor had diarrheal disease at any follow-up measure in the first 24 months of life. Authors reported increased relative abundance of *Bacteroidetes* and enriched communities of inflammogenic taxa among cases (20). While these studies inform hypotheses that fecal microbiota may be implicated in linear growth failure, all were conducted in very small samples of children with co-occurring nutritional deficits. Further work is needed to produce more generalizable results, and to specifically interrogate putative pathways between microbiota and stunting.

That the critical period for linear growth faltering occurs before two years of age presents important methodological challenges to assessing the role of the microbial community in child undernutrition. Even among adults whose microbial communities have long been established, the scientific community has yet to reach a consensus on what comprises a 'healthy' gut population. The post-natal period is the time when a naïve neonatal gut is first exposed to the environment outside of the womb; judging the microbial health of a developing and rapidly diversifying infant gut therefore poses additional challenges to defining a favorable pattern and rate of microbial assembly. To address this constraint, a novel metric called the microbial maturity Z-score (MAZ) was recently developed to assess whether gut populations among undernourished children diverge from an expected pattern of assembly at each month of life from 0-24m, relative to children exhibiting healthy linear and ponderal growth in children living in LMIC (21). Validation of this measure across cohorts of children living in Bangladesh, Malawi and Peru suggests that despite variations in the microbiota, there may be a conserved pattern of acquisition of a set of taxa associated with healthy growth during of this critical postnatal period. When this metric was tested in Bangladesh and Malawi, it was found that microbial maturity was significantly increased among healthy children relative to those with severe or moderate acute malnutrition, with some evidence pointing to the recovery of MAZ scores during nutritional therapy (5,17). This novel metric may therefore be a useful tool for understanding healthy microbial development among vulnerable children, but it has yet to be assessed as an appropriate indicator of linear growth at the population level.

Studies to date have largely comprised translational projects with observational data from very small sample sizes of children selected according to extreme nutritional phenotypes that are then in turn used to inform experimental murine models. Epidemiologic studies powered to assess potential population-level associations between appropriate indicators of gut microbial health and nutritional indices are needed to understand whether this promising new field of research may help to address the burden of childhood stunting. We sought to detect and describe possible associations between child growth and microbial maturity and diversity through 24m of life in a well-characterized large longitudinal birth cohort of infants living with high burden of chronic undernutrition in the Peruvian Amazon. Specifically, we assess whether previously observed associations between MAZ and ponderal growth were detectable in this setting, and whether MAZ and microbial diversity are associated with linear growth.

METHODS.

Study setting. This nested study was conducted in collaboration with the Etiology, Risk Factors, and Interactions of Enteric Infection and Malnutrition and the Consequences for Child Health and Development (MAL-ED) Study, a multi-site longitudinal cohort based in eight settings characterized by high childhood undernutrition and diarrhea. Data was retrieved from one participating MAL-ED site based in Santa Clara de Nanay, a peri-urban riverine community in Loreto province of the Peruvian Amazon. Heavy periods of rain and flooding impact food stability as well as water and sanitation access and quality in this region. Rates of chronic undernutrition are considerably higher than elsewhere in Peru, though acute malnutrition is relatively uncommon. An extensive description of this site has been published (22).

Study design. A high rate of loss to follow-up was anticipated due to frequent travel along the river, resulting in proposed enrollment of 300 children with an aim to retain a birth cohort of at least 200 for the MAL-ED Study. Children were enrolled within 17 days of birth and followed to two years of age between November 2009 and February 2012. Date of birth, sex, birth weight, anthropometrics and details on breastfeeding initiation were recorded at enrollment. To describe patterns of asymptomatic infections and enteric disease, routine stool collection was conducted monthly and additional stool samples collected during each diarrheal episode throughout follow-up. Active surveillance was conducted twice-weekly to provide illness, treatment, breastfeeding practices and dietary intake history, and weight and length were measured monthly to generate calculate anthropometric measures of growth attainment. Details of data collection for MAL-ED are available elsewhere (23–26). Fecal specimens from routine collections at 6, 12, 18 and 24m of age were frozen at -70° C and selected for 16S rDNA profiling.

Specimen analysis. Analysis of routine and diarrheal fecal specimens was conducted according to a unified MAL-ED protocol designed to detect the presence of over 40 enteropathogens. Details of microscopy, bacterial culture methods, immunoassays and amplification methods to identify bacteria, protozoa and viruses across all eight sites have been published (27). Diagnostic analysis generating infection and illness history was conducted at AB Prisma in Loreto, Peru.

Analysis of the gut microbiota from the subsample of 928 stools selected at quarterly intervals was conducted at the Gordon Laboratory at Washington University in St. Louis, Missouri. DNA was isolated from aliquots of pulverized, frozen fecal samples. Fecal samples were homogenized by bead beating for 4 minutes (MiniBeadbeater-96™, Biospec Products; Bartlesville, Oklahoma USA) in a mixture of 250 µL of 0.1 mm-diameter zirconia/silica beads (and a 3.97 mm-diameter steel ball for a subset of samples), 710 µL of 500:210 2X buffer A (200 mM NaCl, 200 mM Tris, 20 mM EDTA):20%SDS, and 500 µL of 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. Samples were then centrifuged at 3,220 x g for 4 min at room temperature. 350 uL of aqueous phase was transferred to a new tube containing 500 uL 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. The tubes were inverted 10 times to mix and then centrifuged at 3220g X 4 min. 250 uL of aqueous phase was transferred to a 96-well plate. DNA was purified from a 100 uL aliquot of each 250 uL crude DNA sample by mixing 100 uL of crude DNA with 400 uL of a 675:45 mix of Qiagen® buffer PM:3M NaOAc, pH5.5 and then passing the mixture through a Qiagen® QIAquick96 plate by centrifugation at 3220g X at least 4 min. Wells were washed twice by passing 900 uL Qiagen® buffer PE through the plate by centrifugation at 3220g X at least 2 min. Quant-iT dsDNA Broad-Range Assay Kits (Invitrogen; Carlsbad, California, USA) was used to quantify the pure DNA.

For each DNA sample, the V4 regions of bacterial 16S rRNA (V4-16S rRNA) genes present were amplified using previously published primers (17) and the following PCR conditions: an initial denaturation step of 2 min at 94°C, followed by 26 cycles of 15 sec at 94°C, 30 sec at 50°C, and 30 sec at 68°C, followed by a final step at 68°C for 2 min. Amplicons were sequenced using an Illumina MiSeq instrument. The resulting 250-nucleotide paired-end reads were trimmed to 200 bases and merged with Flash, and QIIME (v 1.9.0) (28) was used to demultiplex the reads and cluster them into operational taxonomic units (OTU; clusters sharing ≥97% sequence identity). OTU that failed alignment by PyNast (29) were removed from the dataset. Taxonomy was assigned to each OTU using the Ribosomal Database Project (RDP) classifier version 2.4 (30) and the 2013 Greengenes reference database (31). The resulting OTU table contained 32,300±16,453 (mean±sd) reads per sample. Subsequently, OTU that did not have relative abundances of ≥1% in at least one sample or ≥0.1% of the reads in at least two samples were removed from the data. The data were then rarefied to 11,000 reads per sample, and Shannon's diversity index, the total phylogenetic diversity, and the observed number of OTU for each community were calculated as measures of microbial richness and diversity. Samples that did not have at least 11,000 reads were removed from the data.

To characterize the relative maturity of the gut microbiota, we additionally assessed monthly fecal samples from 22 children in the cohort whose anthropometric data never fell below -1.75 standard deviations from the reference mean. A random forests machine learning algorithm was used to predict the ages of each sample from the 22 healthy reference children, taken up to 2 years of age, from the rarefied OTU relative abundances. Feature importance scores were assigned to each OTU using the difference in the mean-squared error of the model when the OTU was included or excluded. One hundred independent runs of the algorithm were performed, and 30 OTU with the greatest mean feature importance scores across all runs were retained to create sparse models. Subsequently, 1,000 unique, randomly selected training sets of 11 of the 22 healthy reference children were generated, and a separate random forests model predicting the ages of samples from these training sets, including only the 30 selected OTU in the sparse set, was generated for each set. Thirty OTU were selected for the sparse random forests model to maintain consistency with similar sparse models constructed for other sites, using the criterion that the number of OTU included should have a mean squared error within 1% of that of optimal number of OTU across 100 implementations of 10-fold cross-validation. Using each model, the age of each sample from all children not in the training set was predicted. For each sample, the mean predicted age across all these models was used as its predicted age. The median and standard deviation of these predicted ages was calculated for each 1-month age bin, and each sample's microbiota-for-age z-score (MAZ) was calculated as the ratio of the difference between that sample's predicted age and the median for the appropriate age bin and the standard deviation for that age bin.

Analytic approach.

Definition of covariates and outcomes. Standardized weight-for-length (WLZ) and length-for-age (LAZ) Z-scores were calculated according to the World Health Organization's Multicentre Growth Reference Study Group guidelines from 2016 (32). Cutoffs of -2 standard deviations were used to classify children as wasted (WLZ) or stunted (LAZ) at each time point under follow-up. Children were classified as severely stunted at or below -3 LAZ. Covariates considered for inclusion in modeling relationships between child growth and gut microbial indices included diarrhea, asymptomatic infection with a known enteropathogen, antibiotic use and infant feeding practices. Diarrheal episodes were defined as occurrence of ≥3 loose stools in a 24-hour period, and asymptomatic enteropathogen exposure was measured by calculating the mean number of pathogens detected in surveillance stools contributed per child at each follow-up measure. Breastfeeding was measured using a first categorical metric to indicate whether children were exposed to breastmilk only (exclusive), mixed milk and other liquids (predominant) or foods (partial), or weaned. Intensity of exposure to breastmilk was also quantified by using the maternal report of number of reported feeds in the past 24 hours to calculate a cumulative, moving average of the number of feeds per child throughout follow-up. Dietary diversity was defined as the cumulative number of seven complementary food groups children had ever been exposed to by each follow-up measure (33). Antibiotic exposure was considered as a continuous variable enumerating the number of days with reported antibiotic exposure per child.

The standardized MAZ score was used to denote microbial maturity at each quarter of life. The primary measure of diversity used in this analysis was the Shannon Diversity Index (SDI), a measure of within-sample bacterial diversity accounting for number and distribution of different species with equal sensitivity to rare and abundant species (34). Secondary outcomes included the CHAO1 (35), which uses a capture-recapture method to enumerate species richness; phylogenetic diversity (36), calculated as the sum of all phylogenetic branch lengths represented within a sample; and Simpson's Diversity (37), the sum of the squared frequency of all OTU within a sample.

Statistical analyses. Longitudinal data analyses were conducted to describe patterns of linear and ponderal growth from birth to two years of age, and sequence plots were employed to visualize transitions between categories based on age normalized (Z-score) categories for LAZ per individual throughout follow-up. Longitudinal associations between linear and ponderal growth and between anthropometrics and microbial indices were assessed using multiple linear regression with a generalized estimating equations approach to adjust for within-child correlation across the monthly or quarterly time points depending on the outcome. Cumulative measures of time-varying covariates were calculated at 6, 12, 18 and 24 months of age for adjustment in final models of microbial maturity, diversity and richness. Associations between anthropometrics and the microbiota were assessed separately at each quarter of life using generalized linear models specifying continuous outcomes. Models were additionally adjusted for LAZ at birth, breastfeeding and complementary feeding exposures, infection and diarrhea history and antibiotic exposure, which were included as a priori confounders depending on the relationship being tested. Models assessing relationships between MAZ and growth were adjusted for breastfeeding exposure, weaning status and anthropometric measure at birth; dietary, illness and treatment variables were excluded from preliminary analyses due to the novelty of this metric and the lack of evidence as to how these factors may affect the particular age-discriminatory OTU comprising the MAZ score. However, due to clear age trends and a wider body of evidence linking additional covariates to microbial diversity, dietary diversity, diarrhea, infection and antibiotic history, and age were additionally included in fully adjusted models assessing relationships between diversity, richness and child growth.

RESULTS.

Description of the population. Two-hundred and seventy-one of the 303 MAL-ED children (89.4%) were included in the Peru Microbiota Collaboration; the remaining 32 children who were not included were lost to follow-up prior to 6m of age. Monthly measurements for 146 boys and 125 girls were used for generation of infection, illness and anthropometric history from 0-24 months. Throughout 6096 months of person-time under follow-up, children contributed 6,011 surveillance stools and 2,440 diarrheal samples to the study. Mean number of anthropometric visits per child was 22.1 from birth to two years of age.

Stool samples at 6, 12,18 and 24m of age were selected for microbial sequencing and generation of maturity and diversity indices. The 271 children contributed a total of 928 samples to this analysis (mean 3.4 samples per child). Throughout follow-up, 93% (250), 85% (231), and 79% (213) of children remained under follow-up at 12, 18 and 24m of age respectively.

Child growth in the Santa Clara cohort. Overall, 181 (66.7%) of the 271 children included in the collaboration were stunted (LAZ<-2) at least once from 0-24m of age. Approximately one in five children (22.1%) in the cohort were severely stunted (LAZ<-3) at least once throughout follow-up. Thirty-four children (12.6%) were stunted at enrollment, which occurred within 17 days of birth, and an additional 90 fell below -2LAZ at least once before 6m of age. While 40% of the 213 remaining under follow-up at 24m of age were stunted, 70% had measured below -2LAZ at one or more of their monthly anthropometric visits (see Table 1). Transitions between categories of stunting severity are shown in Figure 1. We found that children who were stunted at least once before two years of age measured at below -2LAZ on 45% of their visits on average. The majority of children did remain consistent in binary stunting status throughout the first two years of life: the probability that a child would be classified as not stunted, given they were not stunted at the last quarterly measure, was 79%; the probability that a child who was stunted six months prior remained stunted at a given visit was 76%.

While 25 children were wasted (WLZ<-2) on at least one occasion before 2 years of age, mean Z scores in this cohort were higher than the WHO reference for healthy weight-for-length at each quarter of life. Over half of all children were recorded to have WLZ above +2SD at least once before their second birthday. In a model adjusting for age, LAZ at birth, breastfeeding frequency, weaning status, infection and diarrheal history, there was an inverse correlation between WLZ and LAZ scores in this population; children with lower LAZ had higher WLZ, on average (aβ=-0.24, p<0.001). Figure 2a shows linear and ponderal growth trajectories by age; Figure 2b shows the difference in linear growth trajectory stratified by wasting. An interaction term was added to explore whether LAZ trajectories differed significantly between children above and below -2WLZ, and this was found to be statistically significant (p<0.001). After stratification by wasting status, children above -2WLZ decreased in LAZ score at a rate of 0.02SD per month of life (p<0.001) after adjustment for covariates. In contrast, children who were wasted decreased in LAZ score by 0.07SD per month of life (p=0.001).

Microbial indices in the Santa Clara Cohort. Mean maturity, diversity and richness indices at each quarter of life are shown in Table 2. All diversity indices increased significantly by month of age, but this association was not seen for the age-standardized

MAZ score (β=-0.01, p=0.119), as expected. Breastfeeding frequency, calculated as mean number of feeds in last 24 hours, had a significant suppressive effect on all microbial indices, and weaning was significantly associated with increased indices throughout follow-up ($p<0.001$). After adjustment for age and weaning, cumulative dietary diversity score was positively associated with all microbial indices. Associations between microbial indices, age, breastfeeding and dietary varirelativeables are shown in Table 3.

Associations between microbial maturity and growth. No significant association was detected between MAZ and WLZ throughout life (β=-0.05, p=0.118), and this did not change after adjustment for breastfeeding or weaning status (aβ=-0.02, p=0.458) nor when modeled separately at each time point. Mean MAZ scores were lower among stools contributed by wasted children relative to non-wasted children; however, there were only 10 children (15 specimens) meeting these criteria who contributed specimens to the 928 samples in Peru Microbiota Collaboration (Table 4). When we investigated whether MAZ at 6, 12, and 18m of age were predictive of subsequent WLZ scores 1m and 6m thereafter, we found that MAZ was significantly predictive of increased WLZ score 1m but not 6m thereafter. In a model adjusting for WLZ score at current time point, a 1-point increase in current MAZ score was associated with a mean increase of 0.03 WLZ one month thereafter (β=0.03, p=0.035). When breastfeeding frequency and weaning status were accounted for, the effect size was unchanged but the threshold for statistical significance was no longer met (aβ=0.03, p=0.071).

No significant relationship between MAZ and LAZ was observed in the cohort throughout the first two years of life in univariate or adjusted model (β=0.02, p=0.305; aβ=0.03, p=0.156). Given the distinct LAZ trajectory shown among wasted children in

Figure 2b, we investigated whether the relationship between MAZ and LAZ also differed within this subpopulation. Holding age constant, MAZ was found to be positively associated with LAZ among the 10 children (15 observations) who were wasted at the time of quarterly follow-up measures, (β=0.55, p=0.004). After additional adjustment for LAZ at birth, breastfeeding frequency and weaning status, each 1-point increase in MAZ score among wasted children was associated with a mean increase of 0.40LAZ (p=0.013) from 0-24m of age. Finally, we observed that length at birth was significantly associated with lifetime MAZ score (β=0.10, p=0.012); a 1-point increase in LAZ score at birth was associated with a mean increase of 0.11 in MAZ score from birth to 24 after adjustment for breastfeeding frequency, weaning status and diarrhea history (aβ=0.11, p=0.008; Table 5).

Associations between microbial diversity and linear growth in the Peru cohort. We observed a negative association between LAZ score and diversity indices in unadjusted analyses including age as the only covariate, but this was not significant after adjustment for LAZ at birth, breastfeeding and dietary behaviors, and diarrhea and infection history (Shannon: aβ=-0.03, p=0.174; CHAO1: aβ=0.00, p=0.474; PD: aβ=-0.01, p=0.300; Simpson: aβ= -0.19, p=0.118). However, we did find significant evidence that LAZ at birth impacted microbial diversity from birth to two years of age, shown in Table 5. In fully adjusted models, children who were born stunted had reduced lifetime microbial diversity and richness as measured by three of the four metrics assessed. Only eight of the 34 children in the cohort who were born stunted recovered by 24m of age; while this study was not powered to detect significant differences within this subgroup, we did observe lower diversity metrics among those 26 children who were unable to recover growth (defined as attainment of LAZ>-2 by 24m), relative to those who were.

Given the expected changes in diversity over this period of life, we sought to measure specifically whether diversity acquisition was compromised between children of different growth profiles (Figure 3). The apparent increase in diversity seen among those 25 children who were severely stunted, but not born stunted, was explained by the increased proportion of weaned children in this group: 60% were weaned by two years of age, relative to only 27% of the all other children. After adjustment for weaning, there was no significant relationship between severe stunting and diversity acquisition among children who were not born stunted in this cohort. However, children who were born stunted did experience significantly reduced diversity acquisition in the second year of life, after adjustment for covariates (Table 6).

Our observation that breastmilk exposure appears to suppress microbial diversity (Table 3) led us to test the a posteriori hypothesis that children diverge in their diversity trajectories when exposure to breastmilk ceases. We found significant evidence of interaction by weaning, supporting this hypothesis. In the presence of breastmilk, there was no significant reduction in diversity among those children born stunted; however, once weaned, these children exhibited reduced diversity and richness relative to those born at above -2LAZ (Table 6).

DISCUSSION.

This work serves to support the growing field of evidence highlighting differences in gut microbial profiles of children with disparate growth trajectories living in lower and middle-income settings. We describe associations between microbial diversity and maturity with ponderal and linear growth in the largest sample to our knowledge in which these relationships have been interrogated. In contrast to previous work in Bangladesh and Malawi (5,17), we do not report the MAZ metric as a significant indicator of wasting in this population. However while patterns of ponderal growth in this cohort were comparable to other Latin American settings, they are extremely dissimilar to contexts in which MAZ has been used before. Children in the Santa Clara cohort were born considerably above the standard weight for their height, and the prevalence of wasting was very low relative to South Asian or African settings (1,38). We also observed a lower variability in MAZ than expected, with the population mean close to zero for all time points except 18 months when maturity exhibited a dip before recovering by two years of age. Therefore, we caution against the interpretation of these findings as a refutation of the utility of the MAZ marker. Due to the distributions in WLZ and MAZ observed, this population may not be an appropriate one with which to draw inferences about the relationship between microbiome maturity metric and child wasting. Given that we did note a trend in reduced MAZ among the very small number of children contributing samples while wasted, our finding may be a reflection of sample size. Our observation of significant interaction between wasting and stunting is consistent with the literature (38,39). Further, that we observe pronounced linear deficits among children with any wasting history, in conjunction with the detection of a significant relationship between LAZ and MAZ among these children only, lends evidence to the possibility of an underlying relationship between WLZ and MAZ. We did not detect any relationship between linear growth metrics and microbial maturity in the wider cohort, and to our knowledge, no other studies have assessed potential relationships between MAZ and linear growth.

We describe patterns of diversity acquisition in 271 children living in an area of endemic diarrhea and enteropathy and demonstrate significant dietary determinants of this trajectory over 24 months. The presence of breastmilk in infants' diets appears to exert a suppressive effect on all four markers of diversity and richness, while weaning and increasing exposure to a greater number of WHO-defined food groups are correlated with increased diversity and richness metrics thereafter. Our findings are consistent with the wider literature demonstrating the resilience to external events, such as antibiotic challenge and food introduction, that characterize the gut microbiota of breastfed infants, and the subsequent expansion of diversity with exposure to complementary foods (40–48). These observations have led to an evolving hypothesis that healthy infant gut progression is marked by limited diversity in early life (49). Breastmilk selects for the high abundance of only taxa that can metabolize human milk oligosaccharides, most notably *Bifidobacteria*, thus leading to low overall diversity. Authors of a recent review suggest that low diversity driven by the presence of breastmilk is therefore not detrimental, but if the gut community does not diversify after cessation of breastfeeding, it may confer health risks (49). While evidence is largely derived from high-income settings, low microbial diversity in early life has been linked to asthma, allergy and Type I diabetes (50–56), and its association in adulthood with a range of inflammatory, immune, and infectious disease risk has been extensively reviewed (57–60). Laursen et al. call for longitudinal studies specifically employing repeat measures of microbial diversity and dietary intake to further elucidate the rate and timing of microbial progression in the infant gut.

The current study meets these criteria and produces findings consistent with their hypothesis; we detect little variation in microbial outcomes while breastfed, but when exposure to breastmilk ceases we observe rapid diversity acquisition and children begin to diverge in their trajectories. Indeed, we note reduced diversity acquisition among children in one nutritionally vulnerable group: those born at least two standard deviations beneath the standard reference (or born stunted). Specifically, we report that children born stunted experience a distinct pattern of microbial acquisition that is pronounced in the second year of life and after weaning, which results in significantly lower values of diversity and richness than those born above -2 LAZ. Furthermore, we observed higher diversity among children in this subgroup who were able to recover growth and measure above -2 LAZ by two years of age relative to those who remained stunted. While this comparison was done among very few children and was not statistically significant, it further supports the hypothesis that failure to diversify the gut environment beyond the period of breastfeeding denotes vulnerabilities.

Thirteen percent of children in our cohort were stunted at birth, and that LAZ at birth was a significant predictor of subsequent growth. That stunting often begins in utero and has lasting impacts on growth is well documented, and the target period for nutritional interventions has expanded accordingly to prioritize the 1000 days from conception to two years and extend to women of childbearing age (1,61,62). However, research assessing the gut microbiota of children born with prenatal growth faltering is sparse. One exception is the case of necrotizing enterocolitis (NEC), a potentially fatal condition characterized by intestinal pneumotosis, which has been linked to altered gut microbiota among babies born pre-term. Studies in high-income countries have indicated reduced diversity as a correlate of NEC, and recent systematic analyses indicate that probiotics prevent clinical progression and death (63,64). Of the three studies evaluating microbial differences according to linear growth faltering in LMIC settings, one case-control of 20 children included low birth weight babies (20). While they did not find statistically significant differences in diversity they noted increased relative abundance of *Lactobacilli* and *Bifidobacteria* in controls, and of *Bacteroidetes* and inflammogenic taxa in cases. We present evidence that prenatal linear growth faltering is associated with distinct gut microbial metrics enduring over a year beyond birth, and that this apparent compromise is significantly more impactful in weaned children. Our ability to understand how these deficits arise is limited by the absence of maternal variables in this study. Maternal nutritional indices as well as fecal, vaginal and breastmilk samples for microbial sampling would allow us to characterize potential differences in the flora to which children of undernourished mothers are exposed, and may help to further elucidate microbial pathways through which intergenerational stunting occurs. Nonetheless, this data adds a novel element to the evidence of health risks conferred by intrauterine growth restriction, and further supports public health efforts to target nutritional interventions before birth. Specifically, our results suggest that continued breastfeeding may be important to offset potential intestinal risks of being born at low length for age. Continuing breastfeeding to two years receives less emphasis than exclusive breastfeeding in global health messaging, as evidenced by its omission in the World Health Assembly's 2025 nutrition targets (65), and has recently been noted to decline among the poorest populations (66,67). This work, in the context of the growing body of evidence revealing the importance of breastfeeding duration and not just exclusivity (68), should inform nutrition behavior change campaigns to prioritize the promotion of continued as well as exclusive breastfeeding in the first 6 months of life.

We did not identify any significant patterns of gut microbial diversity among children who were born above -2 LAZ and then became stunted later in life. Studies that have assessed and found associations between microbial diversity and linear growth metrics have relied on case control design with quite extreme phenotypes. Furthermore, they do not discriminate between children born stunted and those who later became stunted. That we did not find any microbial markers of postnatal linear growth failure in our cohort may be due to several reasons. First, we did not investigate beta-diversity, nor relative abundances of bacterial taxa, both of which have been reported to differ according to linear growth in the existing evidence. It is possible that alpha-diversity is unaffected among children who become stunted, but key species responsible for nutrient harvest or metabolic or inflammatory responses exhibit distinct progression. Second, this population was characterized by a high toll of chronic undernutrition, as evidenced by the mean LAZ at two years of age at the threshold of stunting. Reassessing these relationships in a less skewed population with a broader spectrum of healthy growth trajectories may be more appropriate for detecting differences between groups. Other limitations of this work include our reliance on 16S rRNA gene sequencing to identify bacterial taxa in the gut. While this methodology has been standard in the literature, its limitations are well documented, including limited precision in identifying bacteria at the species level and risk of bias towards amplifying genes from particular groups over others (69–71). Whole-genome sequencing should be employed for future studies in order to improve precision as well as expand analyses to characterizing metabolomic and proteomic functions of microbial populations in the human gut, which have been shown to be more preserved across geographic settings than bacterial populations (72).

The current study echoes much of the existing literature surrounding linear growth failure, in that it illustrates another mechanism through which prenatal events can set children on a path to growth failure but fails to identify any tangible patterns among children who become stunted after birth. This highlights an ongoing challenge in the field; while stunting that occurs prenatally is still not fully understood, estimates suggest that approximately 80% of the burden occurs in children who are not born with signs of intrauterine growth restriction (61,62). Therefore, while the focus on nutrition interventions for women of childbearing age is of irrefutable importance to promoting child health and development in LMIC, the research community has yet to identify clear markers and intervention targets to address the vast burden of children suffering from stunting without any signs of predisposition at birth. Additional analyses interrogating various characteristics of the gut microbiota that may differ among children with postnatal linear growth failure in multisite cohort studies, such as the one in which the current work was nested, will be invaluable to understanding whether there are actionable targets in the gut microbial community that can help alleviate the worldwide burden of stunting. Nonetheless, this work represents the first large scale birth cohort to longitudinally assess microbial acquisition trajectories during a critical window for child growth and development, and provides evidence that children with intrauterine growth restrictions resulting in short length at birth experience compromised gut diversity that not only endures but is magnified two full years thereafter. More work is needed to confirm and further describe which bacterial populations are most affected, and inform possible interventions such as pre- or probiotic supplementation for children born with existing nutritional vulnerabilities.

Table 1: Anthropometric indices demonstrate low prevalence of wasting but progressive linear growth failure among children in the Peru Microbiota Collaboration

Mean anthropometric indices at each quarter of life from 0-24m among 271 children in the Peruvian Amazon

	Age (months)			
Anthropometric indicators [mean (sd); *95%CI*]	6	12	18	24
Length-for-age Z-score (LAZ)	-1.33 (0.06) *-1.44, -1.22*	-1.56 (0.06) *-1.67, -1.45*	-1.86 (0.06) *-1.98, -1.74*	-1.91 (0.06) *-2.02, -1.79*
Weight-for-length Z-score (WLZ)	1.02 (0.07) *0.89, 1.14*	0.43 (0.07) *0.30, 0.56*	0.15 (0.07) *0.01, 0.29*	0.23 (0.06) *0.11, 0.36*
Stunting Indicators %(n)				
Proportion stunted at each age (LAZ ≤-2)	22.2 (60)	31.5 (78)	42.9 (96)	40.1 (87)
Proportion ever stunted by each age	45.9 (124)	56.1 (139)	69.2 (155)	70.1 (151)
Proportion stunted at last visit who are stunted at this visit	80.0 (48)	78.2 (61)	94.8 (91)	93.1 (81)
Proportion severely stunted at each age (LAZ≤-3)	4.1 (11)	5.3 (13)	10.3 (23)	10.8 (23)
Proportion ever severely stunted by each age	13.3 (36)	17.3 (17)	23.2 (52)	24.4 (52)
Wasting Indicators % (n)				
Proportion wasted at each age	0 (0)	1.6 (4)	2.7 (6)	1.4 (3)
Proportion ever wasted at each age	3.7 (10)	6.7 (17)	9.8 (22)	10.3 (22)

Table 2: Limited variation in microbial maturity and progressive attainment of diversity and richness among children in the Peru Microbiota Collaboration

Mean metrics of microbial maturity, diversity and richness in surveillance stools at each quarter of life among 271 children in the Peruvian Amazon

	Age (months)			
	6	12	18	24
Microbiota maturity [mean (sd); *95%CI*]				
MAZ	0.08 (0.09) *-0.10, 0.25*	0.04 (0.07) *-0.10, 0.17*	-0.25 (0.06) *-0.37, -0.13*	0.03 (0.05) *-0.07, 0.13*
Microbial diversity [mean (sd); *95%CI*]				
Shannon Diversity	1.51 (0.04) *1.42, 1.59*	2.79 (0.06) *2.67, 2.90*	3.89 (0.07) *3.75, 4.02*	4.65 (0.06) *4.54, 4.76*
CHAO1	77.48 (1.74) *74.05, 80.92*	130.61 (2.77) *125.16, 138.06*	191.40 (3.79) *183.93, 198.87*	237.30 (3.83) *22.73, 244.85*
Phylogenetic diversity	5.88 (0.12) *5.64, 6.12*	10.24 (0.20) *9.85, 10.62*	14.13 (0.26) *13.61, 14.65*	17.59 (0.27) *17.06, 18.12*
Simpsons Diversity	0.40 (0.01) *0.38, 0.43*	0.67 (0.01) *0.65, 0.69*	0.82 (0.01) *0.80, 0.84*	0.90 (0.01) *0.89, 0.91*

Table 3: Breastmilk exposure is significantly associated with reduced microbial diversity, richness and maturity, which increase in conjunction with weaning and increasing dietary diversity

Associations between breastfeeding, weaning, dietary exposure and microbial indices from 0-24m of age among 271 children in the Peruvian Amazon.

	Metrics of gut microbial maturity & diversity Mean (SD); *95%CI*				
	MAZ	Shannon	CHAO1	Phylogenetic Diversity	Simpson
Age (months)	-0.01 (0.01) *0.119*	0.18 (<0.01) *<0.001*	8.86 (0.23) *<0.001*	0.64 (0.02) *<0.001*	0.03 (<0.01) *<0.001*
Breastfeeding frequency* (Mean # feeds/past 24hr)	-0.03 (0.01) *0.001*	-0.03 (0.01) *0.002*	-1.63 (0.49) *0.001*	-0.10 (0.04) *0.006*	-0.01 (<0.01) *0.003*
Weaning status* (Weaned/not weaned)	0.31 (0.08) *<0.001*	0.57 (0.08) *<0.001*	28.75 (4.40) *<0.001*	1.69 (0.30) *<0.001*	0.04 (0.02) *0.007*
Dietary diversity** (0-7 WHO food groups)	-0.04 (0.02) *0.013*	0.14 (0.02) *<0.001*	3.80 (0.99) *<0.001*	0.34 (0.07) *<0.001*	0.04 (<0.01) *<0.001*

**Estimates are age-adjusted for diversity indices but not for MAZ.*
***MAZ estimate adjusted for weaning and breastfeeding frequency; diversity estimates are additionally adjusted for age*

Table 4: Children with wasting show delayed microbial maturation in the Peru Microbiota Collaboration.

Mean MAZ in 15 samples contributed by children while wasted (n=10), relative to 913 samples contributed among non-wasted children (n=271).

	MAZ *Mean (SD); 95%CI*	
Age	Wasted	Non-wasted
6	NA	0.19 (0.11) *-0.03, 0.40*
12	-0.42 (0.37) *-1.15, 0.30*	0.05 (0.07) *-0.09, 0.19*
18	-0.58 (0.24) *-1.11, -0.11*	-0.24 (0.07) *-0.36, -0.12*
24	-0.30 (0.67) *-1.61, 1.02*	0.03 (0.05) *-0.07, 0.13*

Table 5: Significant reductions in microbial diversity and richness associated with low length-for-age at birth

Results of multivariable linear regression testing associations between length at birth and microbial maturity, diversity and richness from 0-2 years of age among 271 children in the Peru Microbiota Collaboration.

	Metrics of gut microbial maturity & diversity Mean (SD); *95%CI*				
	MAZ	Shannon	CHAO1	Phylogenetic Diversity	Simpson
LAZ at birth	0.11 (0.04) *0.008*	0.07 (0.03) *0.017*	3.57 (1.68) *0.034*	0.23 (0.12) *0.056*	0.01 (0.01) *0.078*
Stunted at birth	-0.12 (0.12) *0.296*	-0.19 (0.08) *0.027*	-9.75 (4.81) *0.043*	-0.73 (0.35) *0.037*	-0.03 (0.02) *0.111*
Recovered (n=8)	Ref				
Remained stunted (n=26)	0.05 (0.22) *0.824*	-0.26 (0.21) *0.215*	-5.68 (12.58) *0.652*	-0.47 (0.79) *0.548*	-0.06 (0.04) *0.085*

MAZ estimates adjusted for breastfeeding frequency and weaning; diversity estimates additionally adjusted for age, dietary diversity, and diarrhea & infection history

Table 6: Pronounced reductions in gut diversity and richness in the second year of life and by weaning status among children born stunted

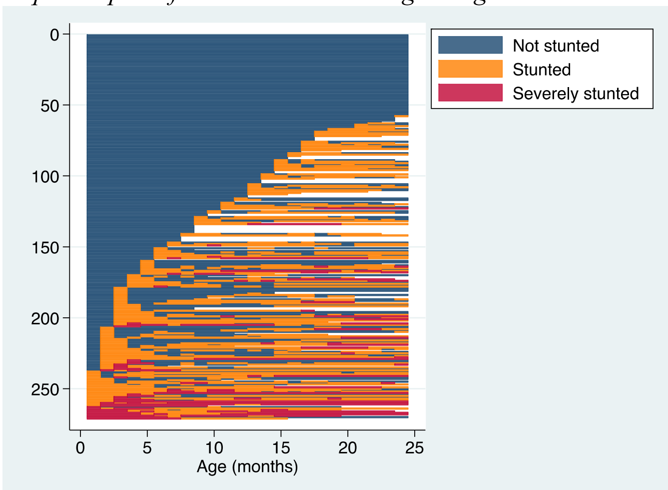
Results of multivariable linear regression models testing for interactions between being born stunted and i) year of life or ii) weaning status in the relationship between being born stunted and gut microbial diversity among 271 children in the Peru Microbiota Collaboration

	Metrics of microbial diversity & richness Mean (SD); *95%CI*			
	Shannon	CHAO1	Phylogenetic Diversity	Simpson
Stunted at birth	-0.02 (0.11) *0.888*	-2.60 (6.1) *0.670*	-0.07 (0.43) *0.877*	<0.01 (0.02) *0.820*
Year of life				
Year 1 (0-12m)	Ref			
Year 2 (13-24m)	-0.29 (0.12) *0.013*	18.78 (6.4) *0.003*	0.67 (0.43) *0.118*	0.04 (0.02) *0.067*
Interaction term *(born stunted*year of life)*	-0.37 (0.15) *0.012*	-15.34 (8.05) *0.057*	-1.42 (0.54) *0.009*	-0.04 (0.03) *0.146*
Stunted at birth	-0.04 (0.10) *0.684*	-3.49 (5.40) *0.519*	-0.27 (0.38) *0.481*	-0.009 (0.02) *0.625*
Weaning status				
Exclusively, predominantly or partially breastfed	Ref			
Weaned	0.74 (0.09) *<0.0001*	31.76 (4.70) *<0.0001*	2.10 (0.32) *<0.0001*	0.07 (0.17) *<0.0001*
Interaction term *(born stunted*weaning status*)	-0.55 (0.17) *0.001*	-23.04 (9.19) *0.012*	-1.68 (0.62) *0.007*	-0.06 (0.03) *0.079*

Models are adjusted for age, breastfeeding frequency, dietary diversity, diarrhea and infection history; year of life additionally included as a potential confounder in model assessing evidence for interaction by weaning.

Figure 1: High consistency in stunting classification throughout first two years of life in the Peru Microbiota Collaboration

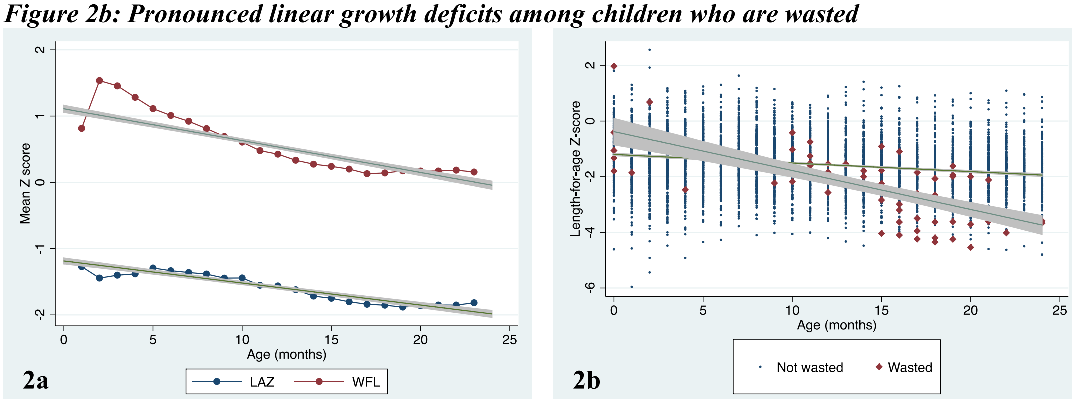
Sequence plot of 271 children showing changes between stunting categories from 0-24m



Each child represented as a different row on the Y-axis; white denotes loss to follow-up.

Figure 2: Trends in linear and ponderal growth from birth to two years of age in the Peru Microbiota Collaboration

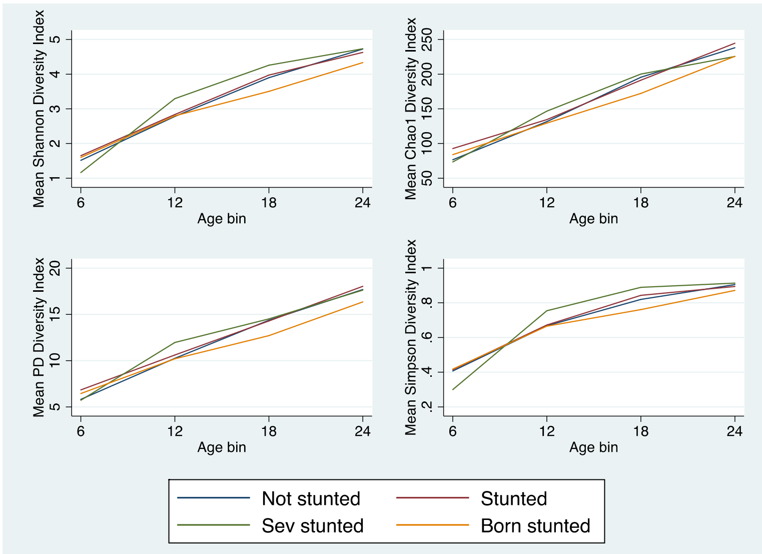
Figure 2a: Low length for age and high weight for length at baseline, with steady reductions from 0-24m
Figure 2b: Pronounced linear growth deficits among children who are wasted



Linear prediction lines shown with 95%confidence intervals shaded in grey

Figure 3: Reduced diversity and richness acquisition among children born stunted relative to other growth profiles

Diversity and richness acquisition trajectories shown by stunting status for 271 children in the Peru Microbiota Collaboration.



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Chapter 5: Diarrhea as a cause and consequence of reduced gut microbial diversity in children in Peru

ABSTRACT.

Background. Detrimental effects of diarrhea on child growth and survival are well-documented in vulnerable populations, but mechanisms remain poorly understood. Recent evidence demonstrates that gut microbiota influence infection and growth in early life, but most has been generated in high-income settings or with small sample sizes. We assessed the bi-directional associations between diarrhea and bacterial diversity among infants of differing growth profiles from a longitudinal birth cohort with high burden of undernutrition and diarrheal disease in Peru.

Methods. Infection and illness history were ascertained through active surveillance and monthly fecal collection from 271 children 0-24m of age. Fecal samples (n=928) at quarterly intervals were selected for identification of fecal microbiota using polymerase chain reaction (PCR) primers detecting 16S ribosomal RNA and generation of indices of diversity and richness (Shannon, Simpson, CHAO1 and phylogenetic diversity). Bi-directional associations between diarrhea and gut diversity were investigated using multiple linear regression with adjustment for within-child correlations, age, breastfeeding, infection and antibiotic history, and dietary diversity. We conducted secondary analyses to test whether possible impacts of diarrhea on the microbiota differed based on stunting or weaning status in the full cohort, or endured beyond one month in a subset of children without recent diarrhea or antibiotic exposure (n=71).

Results. By two years of age, 96% of children experienced diarrhea. Odds of being severely stunted increased by 8% with each additional diarrheal episode throughout the first two years of life (OR=1.08; $p<0.001$). Cumulative diarrheal frequency, duration and severity were associated with significant reductions in microbial indices ($p<0.05$), some of which were still detectable beyond one month. We found significant interaction between stunting status and diarrheal exposure: children who were born stunted experienced greater diversity insults per diarrheal episode than those children who were not stunted (Interaction: Shannon β =-0.04, p=0.037; Simpson β =-0.01, p=0.032). Time elapsed since last diarrheal exposure was associated with recovery of Shannon (β =0.02, p=0.03) and phylogenetic diversity (β =0.11, $p<0.01$) and we detected evidence that this regeneration process was significantly slower among severely stunted children. Lower diversity was associated with subsequent diarrheal incidence, with a 1-unit increase in the Shannon and Simpson's Diversity scales at 6m corresponding to a mean reduction of 1.3 and 3.4 diarrheal episodes from 6-24m of age, respectively.

Conclusions. In a population experiencing the classical cycle of diarrhea and undernutrition we report evidence that disruptions to the gut bacterial community may be implicated in driving the repeated cycle of diarrheal disease experienced by vulnerable children during a critical period for growth and development. This is the largest cohort study to date that interrogates these relationships longitudinally.

INTRODUCTION.

The cycle between diarrhea and undernutrition is well described, but remains poorly understood. Children in economically disadvantaged settings with a high burden of enteric infections and diarrhea experience compromised growth trajectories in early life (1), resulting in impaired immunity and increased vulnerability to future illness (2–7). These conditions continue to account for a vast proportion of childhood deaths: global mortality estimates for children under 5 years of age (U5) attribute 700,000 deaths to diarrhea (8), and over 3 million deaths to underlying nutritional deficiencies (9) annually. Mortality is not the only consequence; diarrhea is the leading cause of long-term disability among children under 5 years of age worldwide due to the consequences of repeated disease for cognition and school-performance, intestinal function, child growth and overall developmental potential (10–14). New estimates accounting for these sequelae increased the number of disability-adjusted life-years lost to diarrhea among U5 by nearly 40% to over 55 million annually (13), and targeting nutritional shortfalls and diarrheal disease have been highlighted as crucial to averting the loss of human potential in 200 million children worldwide (15). The persistence of this cycle in the face of various child health interventions is therefore troubling (16), and recent research efforts have focused on elucidating underlying mechanisms of intestinal health that may be crucial to interrupting it.

Specifically, a growing body of evidence implicates the integrity of gut microbial populations in both enteric health and child growth. Gut microbiota are crucial to achieving healthy metabolism and digestion as well as modulating host defense against pathogens (17,18) and therefore may be implicated in the interplay between infection, immunity and undernutrition in early life. Indeed, a growing body of evidence links the gut microbiota to undernutrition. Gut bacterial populations can promote dietary energy harvest, amino acid synthesis and micronutrient absorption (19–22), and weight loss can be induced by inoculation of germ-free mice with gut flora from undernourished children (23). Recent studies have also shown that diversity and composition of the gut microbiota differ among undernourished children in lower and middle-income countries (LMIC), though most were conducted in children with severe acute malnutrition (SAM) and very few have directly assessed relationships between gut flora and linear growth (24–28).

Studies have also demonstrated changes to particular species and overall gut microbial diversity and richness before, during and after diarrhea. These relationships have most comprehensively been described in the case of *Clostridium difficile* colonization occurring after antibiotic-driven disruptions to gut flora (29). Consequences are severe and can be life-threatening, but may be averted by fecal transplant to restore bacterial populations in the gut (30). Comparison of fecal composition between cases and controls confirms marked reductions in overall microbial diversity among individuals affected, and an observed dose-response in samples from patients with recurrent *C. difficile* suggests that disruptions may be sustained or additive over time (31). A limited number of studies have also identified differences in gut bacterial communities among adults experiencing travelers' diarrhea (32) and norovirus infection (33) in the United States.

Less is known about the relationship between diarrhea and the gut microbiota among individuals residing in LMIC, especially in young children vulnerable to growth insults and other long-term morbidities. One study analyzed fecal samples during and after infection among nine children aged 2-3 years old with confirmed cholera, observing reductions in commensal bacteria and an increased prevalence of harmful *Proteobacteria* (34). Another found a significant shortfall in a standardized microbial maturity score among children during and up to one month after a diarrheal episode, but not beyond (25). While these have been important contributions to evolving hypotheses, they have been conducted with very small sample sizes of fewer than 20 children. One notable exception is a secondary analysis of 922 fecal samples from the Global Enterics Multicenter Study (GEMS) comparing cases of moderate-to-severe diarrhea to controls (35), in which investigators report consistently reduced bacterial diversity and distinct taxonomic composition in diarrheal stools contributed from birth to 5 years of age (36).

Among children suffering multiple episodes of diarrhea in early life, microbiota may therefore be profoundly affected, perhaps in a manner that could help explain the long-term consequences of repeated illness in childhood. The case-control and often cross-sectional design of these studies however limits their ability to detect whether diarrhea-associated differences in microbial composition and diversity are apparent after symptoms subside, and to characterize potential changes to the these metrics over time among children experiencing a high burden of disease in early life. To our knowledge no study has been conducted in LMIC settings to assess whether children with variable diarrheal disease history differ in their microbial succession during this critical window for child growth and survival. The current study leverages data from a longitudinal birth cohort to explore bi-directional associations between diarrheal disease and microbial diversity and richness among 271 children from birth to two years of age living in the Peruvian Amazon. We additionally aim to measure whether potential impacts of diarrheal insults on the microbial community endure over time, and whether relationships differ according to nutritional indices and complementary feeding practices, in order to understand potential interactions between nutrition, diarrheal disease and the developing infant gut microbiota in LMIC.

METHODS.

Study setting and design. This study was conducted within the Peru-Microbiota collaboration, a partnership between Johns Hopkins University, Washington University and Asociación Benéfica PRISMA in Peru. Data was ascertained from the Peru site of the Etiology, Risk Factors, and Interactions of Enteric Infection and Malnutrition and the Consequences for Child Health and Development (MAL-ED) Study, a longitudinal study based in eight settings characterized by high childhood undernutrition and diarrhea (37). The research site in Peru is located in the lowlands of the Amazon basin, a region consistently demonstrating some of the poorest health and economic indices in the country. Access to water, sanitation, and livable wages is markedly lower than elsewhere in Peru, and diarrheal disease prevalence and under-five mortality are nearly three-times the national average (38).

A birth cohort of 303 children was enrolled in Santa Clara de Nanay between November 2009 and February 2012. Details of data collection are reported elsewhere; briefly, date of birth, sex, breastfeeding initiation information and anthropometrics were collected at enrollment, which occurred within 17 days of birth. Children contributed monthly surveillance stool samples for generation of asymptomatic infection history, and were visited twice-weekly for active surveillance of diarrhea which if present resulted in additional specimen collection. Active surveillance visits also ascertained illness and treatment (antibiotic) history, breastfeeding practices and dietary intake. Weight and length were measured monthly to generate calculate anthropometric measures. Analysis of routine and diarrheal stool specimens was conducted in the satellite laboratory in Iquitos, Peru according to unified MAL-ED protocol. Details of microscopy, bacterial culture methods, immunoassays and amplification methods to identify bacteria, protozoa and viruses across all eight sites have been published (39).

In 2016 the Peru Microbiota Collaboration was established to conduct analyses of gut microbiota among children enrolled in MAL-ED Peru. Two-hundred and seventy-one of the 303 children initially enrolled contributed surveillance stools at 6, 12, 18 and 24m of age were selected for microbial analysis by the Gordon Laboratory at Washington University in St. Louis, Missouri. DNA was isolated from aliquots of pulverized, frozen fecal samples. Fecal samples were homogenized by bead beating for 4 minutes (MiniBeadbeater-96™, Biospec Products; Bartlesville, Oklahoma USA) in a mixture of 250 µL of 0.1 mm-diameter zirconia/silica beads (and a 3.97 mm-diameter steel ball for a subset of samples), 710 µL of 500:210 2X buffer A (200 mM NaCl, 200 mM Tris, 20 mM EDTA):20%SDS, and 500 µL of 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. Samples were then centrifuged at 3,220 x g for 4 min at room temperature. 350 uL of aqueous phase was transferred to a new tube containing 500 uL 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. The tubes were inverted 10 times to mix and then centrifuged at 3220g X 4 min. 250 uL of aqueous phase was transferred to a 96-well plate. DNA was purified from a 100 uL aliquot of each 250 uL crude DNA sample by mixing 100 uL of crude DNA with 400 uL of a 675:45 mix of Qiagen® buffer PM:3M NaOAc, pH5.5 and then passing the mixture through a Qiagen® QIAquick96 plate by centrifugation at 3220g X at least 4 min. Wells were washed twice by passing 900 uL Qiagen® buffer PE through the plate by centrifugation at 3220g X at least 2 min. Quant-iT dsDNA Broad-Range Assay Kits (Invitrogen; Carlsbad, California, USA) was used to quantify the pure DNA.

For each DNA sample, the V4 regions of bacterial 16S rRNA (V4-16S rRNA) genes present were amplified using previously published primers (25) and the following PCR conditions: an initial denaturation step of 2 min at 94°C, followed by 26 cycles of 15 sec at 94°C, 30 sec at 50°C, and 30 sec at 68°C, followed by a final step at 68°C for 2 min. Amplicons were sequenced using an Illumina MiSeq instrument. The resulting 250-nucleotide paired-end reads were trimmed to 200 bases and merged with Flash, and QIIME (v 1.9.0) (40)was used to demultiplex the reads and cluster them into operational taxonomic units (OTU; clusters sharing ≥97% sequence identity). OTU that failed alignment by PyNast (41) were removed from the dataset. Taxonomy was assigned to each OTU using the Ribosomal Database Project (RDP) classifier version 2.4 (42) and the 2013 Greengenes reference database (43). The resulting OTU table contained 32,300±16,453 (mean±sd) reads per sample. Subsequently, OTU that did not have relative abundances of ≥1% in at least one sample or ≥0.1% of the reads in at least two samples were removed from the data. The data were then rarefied to 11,000 reads per sample, and Shannon's diversity index, the total phylogenetic diversity, and the observed number of OTU for each community were calculated as measures of microbial richness and diversity. Samples that did not have at least 11,000 reads were removed from the data.

Analytic approach.

Definition of covariates. Diarrhea was defined as occurrence of ≥3 loose stools in a 24-hour period, and distinct episodes classified as separated by either a pathogen negative stool or a period of ≥14 days since last diarrheal stool. The number of distinct episodes experienced by each child from enrollment to each outcome measure was analyzed as a cumulative, time-varying indicator of diarrheal frequency. Severity of episodes was assessed using a Community Diarrheal Assessment (CODA) score, with each episode assigned a score out of 15 points based on maternally reported presence and duration of fever, vomiting, anorexia, liquid stools, and the maximum number of stools in a 24-hour period at any time during the episode (44). Adjustment for potential confounders and assessment of interactions was conducted using various illness, treatment, dietary and growth indicators. Asymptomatic infections were considered using a cumulative time-varying value denoting the mean number of enteropathogens identified per surveillance stool contributed at each point of follow-up. Antibiotic exposure was included as a continuous variable representing the number of days with any exposure in a child's life. Breastfeeding frequency was considered as a continuous, cumulative average of the number of times the child was brought to the breast in the last 24 hours. Breastfeeding was also considered as a categorical variable indicating exclusive, predominant (other liquids) or partial (foods including solids) breastfeeding. Children were categorized as fully weaned from the day after their last reported breastmilk exposure. Dietary diversity was measured continuously as the number of 7 WHO food groups that a child had ever ingested at each time of follow-up (45). Standardized length- and weight-for-age Z-scores were calculated at each month of life using the World Health Organization's Multicentre Growth Reference Study Group guidelines (46). Children were classified as "born stunted" if their baseline measure was below -2 LAZ. Severe stunting was defined as LAZ measuring at or below -3 standard deviations.

Microbial diversity and richness were assessed using four distinct metrics calculated using OTU identified in fecal samples. The Shannon Diversity Index (SDI) (47) accounts for the number and distribution of different species, and Simpson's Diversity (48) describes the sum of the squared proportional abundances of all OTU within a given sample. Both are measures of alpha-diversity, or within-sample bacterial diversity, however the Shannon index was defined as the primary diversity metric a priori due to its equal sensitivity to rare and abundant species and because it is more widely used in the literature therefore useful for consistency and ease of comparison. Bacterial richness was measured by CHAO1 (49), which uses a capture-recapture method to enumerate species richness and phylogenetic diversity (50), expressed as the sum of all phylogenetic branch lengths represented within a sample.

Statistical analyses. The impact of diarrheal frequency, duration and severity on subsequent microbial metrics throughout the first two years of life was estimated and tested using multiple linear regression (MLR) with a generalized estimating equations approach to adjust for within-child correlation over time. We investigated whether associations between diarrheal disease and microbial metrics endured over time using two distinct approaches. First, we examined time since last diarrheal insult as a predictor of subsequent microbial indices using the same methodology as described above. Second, we repeated our analyses in a sub-cohort of children who were able to contribute stools at quarterly intervals at least one month after most recent diarrheal or antimicrobial perturbation. All models assessing relationships between diarrhea and microbial indices were adjusted for age, breastfeeding frequency and category, dietary diversity, antibiotic exposure and pathogen pressure.

Across the full cohort, we assessed whether relationships between diarrheal disease and microbial metrics differed according stunting and weaning status. We hypothesized that *i) stunted children may be more vulnerable to intestinal insult*, and ii) *that potential changes to the microbiota would be pronounced in the absence of breastmilk*. For each of these analyses, interaction terms were added to the original MLR to assess whether relationships statistically differed across four categories of stunting (not stunted, stunted, severely stunted, born stunted) or weaning status (binary yes/no variable) across the population.

Finally, we assessed whether diversity metrics at 6, 12, or 18m of age were associated with the number of subsequent diarrheal episodes experienced in infants' lives. To do so, we ran MLR models separately at each time point and therefore excluded adjustment for within-child correlations, but retained all other potential confounders.

RESULTS.

Description of diarrhea in the population. Throughout follow-up, 146 boys and 125 girls contributed 6,3031.4 months of person-time and a total of 6,011 surveillance stools and 2,440 diarrheal samples to the study. Seventy-seven percent of diarrheal samples

(1887) were pathogen-positive, with up to 7 distinct enteropathogens detected in a single sample. Two-hundred and sixty children (95.9%) experienced diarrhea during follow up. Mean time to first episodes was 5.3 months, with a median of 4.6 months of age. By two years of age, children experienced a mean of 8.8 diarrheal episodes lasting on average 4 days each. Most common enteropathogens identified in diarrheal stools were *Campylobacter spp* (32%), norovirus (27%), *Giardia spp* (25%), entero-aggregative *E. coli* (17%), and *Cryptosporidium* (10%). Time to first exposure and diarrheal prevalence at each age are shown in Figure 1; indices of diarrheal exposure and microbial diversity in each quarter of life are shown in Table 1. Figure 2 illustrates the increase in microbial indices with age from birth to two years of age.

Description of covariates in the population. Children experienced a high prevalence of asymptomatic infections: 44% of surveillance stools were infected with at least one enteropathogen, and up to 6 pathogens were detected in a single sample by 2 years of age. Ninety-seven percent (262) of children in the cohort were exposed to antibiotics, with an average of 53 days of exposure in the first two years of life. Children underwent a mean of 9.3 distinct courses of treatment lasting an average of 6 days each.

All 271 children in the cohort received breastmilk. Mean duration of exclusive breastfeeding was 41 days. However, switching back to exclusively breastfeeding for some period of time even after introduction of other liquids or foods was a common practice in this population, and the mean number of days with exclusive breastfeeding from 0-24m of age was 94. Mean age at introduction of solids and non-human milk were 144 days (4.8m) and 172 (5.6m) days of age, respectively, and children were weaned at the average age of 593 days (19.5m). No children in the cohort were still exclusively breastfed at 6m of age, and very little variation in breastfeeding habits were observed (See Table 1). By two years of age, 69% of children were exposed to all seven WHO complementary food groups, and all but one child were exposed to at least four of them (meeting a minimum dietary diversity standard (45)). Overall, 181 (66.8%) of the 271 children included in the microbiota collaboration were stunted (LAZ<-2) and one in five children (22.1%) in the cohort were severely stunted (LAZ<-3) at least once throughout follow-up. Thirty-four children (12.6%) were stunted at enrollment, which occurred within 17 days of birth. Twenty-five of the 60 children who were severely stunted in the cohort were born stunted.

Diarrhea, breastfeeding and growth in MAL-ED Peru. Diarrhea incidence increased with age in the first year of life, and decreased thereafter (Figure 1B). Due to the uniformity in breastfeeding habits across the cohort we could not model the relationship between diarrhea and breastfeeding according to the WHO recommended threshold of 6 months of age. Instead, effects were stratified by year of life due to greater variation in breastfeeding habits after 12m of age (see Table 1). After adjustment for age and breastfeeding frequency, the odds of experiencing diarrhea was significantly increased in all categories relative to children who were still exclusively breastfed (Predominant OR: 2.5, p=<0.001; Partial OR: 2.4, p<0.001; Weaned OR: 3.3, p=0.011) from 0-12m of age. In the second year of life, children who were weaned had higher odds of experiencing diarrhea than those children who were still partially breastfed (OR=1.2, p=0.045).

Models assessing the impact of diarrhea on growth were adjusted for age, breastfeeding intensity and category, LAZ at birth, and pathogen pressure. Throughout the first two years of life, each additional diarrheal episode experienced was associated with a reduction in 0.01 LAZ (β= -0.01; p=0.002), after adjustment for age, breastfeeding frequency and category, and LAZ at birth. Odds of being severely stunted increased by 8% with each additional diarrheal episode throughout the first two years of life (OR=1.08; p<0.001) throughout the cohort.

Impacts of diarrhea on microbial diversity and richness. All metrics of diversity and richness increased significantly with age per month of life throughout follow-up (Shannon β=0.18, CHAO1: β=9.0, PD: β=0.65, Simpson β=0.03; p<0.001 for all indices). Frequency of exposure to breastmilk had a suppressive effect on diversity and richness, while weaning and dietary diversity significantly increased all measures (data presented in Chapter 4 of this dissertation).

Table 2 describes associations between diarrheal disease history and microbial metrics from birth to 24m of age among 271 in the Santa Clara cohort. Cumulative diarrheal frequency had a significantly negative association with all four diversity indices after adjustment for age, breastfeeding and dietary factors, pathogen pressure and antibiotic exposure. Further, we found significant interaction between stunting status and diarrheal exposure: children who were born stunted experienced greater diversity insults per diarrheal episode than those children who were not stunted (Interaction terms, Shannon: β =-0.04, p=0.037; Simpson: β =-0.01, p=0.032). This was not seen among measures of bacterial richness (CHAO1: β =-1.21, p=0.239; PD: β =-1.0, p=0.179). Mean diarrheal duration and severity were significantly associated with reduced microbial diversity and richness throughout life (see Table 2), but there was no significant evidence that the relationship between diarrheal duration and severity and diversity indices differed by stunting or weaning status (p>0.5 for all interaction terms).

Finally, we modeled whether time since last diarrheal episode was associated with reduced diversity across the full cohort of 271 children. We observed that the mean number of days since last reported episode were higher in year two than in year one (97 vs 49 days, respectively), during which the odds of experiencing diarrhea was lower (Figure1B). Given that this coincides with the marked increase in diversity in year two, we additionally adjusted these analyses for year of life. Each additional month of life since experiencing diarrhea was significantly associated with a mean increase of 0.02 points on the Shannon diversity scale and 0.11 points in phylogenetic diversity ($p=0.03$ and $p=0.008$, respectively). We additionally detected evidence that severely stunted children exhibited a significantly weaker association between time since diarrhea and increased microbial richness (Interaction terms: CHAO1: $\beta= -4.12$, $p=0.040$; PD: $\beta= -0.28$, $p=0.044$) than those measuring at above -3 LAZ. While this negative trend was also apparent for diversity measures, it was not statistically significant (Shannon $\beta= -0.06$, $p=0.105$; Simpson $\beta=-0.005$, $p=0.488$).

To further investigate whether associations between diarrhea and reduced diversity endure over time, we replicated analyses among children who had not experienced diarrhea or antibiotic exposure for at least one month before contributing stool samples. A total of 71 children (39 boys and 32 girls) contributed 430 diarrheal samples and 1840 surveillance stools for the calculation of illness and infection history, and 254 samples for 16S microbial analysis after being free of diarrheal or antimicrobial perturbation for at least 30 days. Indices of diarrheal disease, microbial diversity and richness, and illness and dietary covariates for this sub-cohort are shown in Supplementary Table 1. Children in this group were similar to the broader cohort, with the exception of reporting fewer diarrheal episodes and days with antibiotic exposure relative to the broader cohort.

We observed a negative relationship between diversity and number of diarrheal episodes in this group, but this was only statistically significant for the Shannon index (Figure 3; Table 3). Each additional diarrheal episode experienced was associated with a reduction in 0.05 points on the Shannon Diversity Index at least one month after insult. Similarly, severity of episodes exhibited a negative trend on all indices, but this only met the threshold of statistical significance for the Shannon Index, which was reduced by an average of 0.01 per one-point increase in the CODA severity score. In contrast, we found that mean duration of reported episodes was significantly associated with reduced microbial diversity and richness after lagging exposures, with each additional day of diarrhea associated with reductions Shannon, CHAO1 and phylogenetic diversity scores that endured >1m after symptoms resolved.

Impacts of microbial diversity and richness on subsequent diarrhea. We found significant evidence that reduced microbial diversity and richness was predictive of more frequent subsequent diarrhea in the full cohort of 271 children. At 6m of age, each additional point on the Shannon and Simpson's diversity metrics was associated with a mean reduction of 1.3 and 3.4 diarrheal episodes from 6-24m of age, respectively (Table 4). At one year of life, higher Shannon, CHAO1 and phylogenetic diversity scores were significantly associated with reduced subsequent diarrhea. While this negative trend was seen at 18m of age, it was not statistically significant.

DISCUSSION.

We describe longitudinal, bi-directional associations between four metrics of bacterial diversity and diarrheal disease in a large birth cohort with a high burden of undernutrition and enteric infections. Patterns between covariates in this cohort were consistent with the wider body of literature demonstrating the protective effects of breastfeeding on the risk of diarrheal disease (51), and the detrimental associations between diarrhea and subsequent child growth (52,53). In a population illustrating the classical cycle of diarrhea and undernutrition we report evidence that disruptions to the gut bacterial community may be implicated in driving the repeated cycle of diarrheal disease experienced by vulnerable children during a critical period for growth and development.

Diarrheal frequency, duration and severity were all significantly associated with reductions in gut microbial diversity and richness. These findings are complementary to a case-control study of 992 children comparing stools of children with moderate to severe diarrhea in West Africa and South Asia enrolled in the GEMS study to non-diarrheal controls. This work, which comprises the largest sample size in a study assessing this relationship in LMIC, demonstrated that diarrheal samples consistently exhibit reduced Shannon diversity relative to controls (36). Our study uses a longitudinal design to demonstrate that these disparities extend beyond the diarrheal episode itself, and are apparent among non-diarrheal samples contributed by children with greater lifetime history of disease. This is also consistent with recent findings from studies conducted among children in Nicaragua and Vietnam. In the former, samples were collected during and 2 months after diarrheal disease among 25 Nicaraguan children of mean age 21 months (54). Authors report no increase in diversity or richness in the 2 months following diarrhea, which is counter to the acquisition curves expected in the developing gut. When separated by pathogen positivity, they further noted that children with pathogen-free diarrhea did exhibit increases in phylogenetic diversity and species richness, whereas those with enteropathogens detected in diarrheal samples did not. In Vietnam, authors identified four distinct profiles of the gut assembly among 55 samples from controls and 145 samples from diarrheal hospital patients under 5 years of age (55), two of which were nearly exclusively comprised of diarrheal cases. While they did not find a clear pattern of Shannon diversity aligning with diarrheal symptoms, their classification of particular taxa showed the degree of divergence from the healthy gut community to be correlated with diarrheal output and hospital duration, implying that severity of disease may be associated with microbial disruption. Authors further observed a depletion of taxa that produce short chain fatty acids, and postulated that this may contribute to shortages in absorption and metabolic energy that can help explain long term sequelae of diarrheal disease.

We also report evidence that apparent impacts on diversity endure beyond one month after diarrheal exposure in a subset of children, and that time elapsed since most recent diarrheal episode was associated with regeneration of the diversity and richness metrics across the full population. This finding may appear at odds with prior observations that the gut microbial community exhibits transient changes followed by apparent recovery one month after diarrheal insult in Bangladesh (25,56). In these studies, patients with severe disease were enrolled and their recovery was characterized after a single, moderate to severe episode either cholera or enterotoxigenic *Escherichia coli* (ETEC). In our cohort, the magnitude of gains in microbial metrics associated with a one-month recovery period was similar to the magnitude of depletion associated with a single episode. Therefore, these findings do not necessarily conflict; the microbial community may recover within one month if the exposure is a single episode of diarrhea. Our data however describes the impacts of repeated disease with multiple episodes of infection and symptoms throughout life, which may explain the more lasting impacts observed.

Slower recovery in diversity and richness was observed among children who were severely stunted. As such, children experiencing multiple episodes in the first two years of life may risk experiencing additive insults to microbial indices that are already diverging from their anticipated trajectory, and this may be even more consequential among children suffering from severe growth deficits. Furthermore, we found that high-risk subsets of children were more susceptible to microbial perturbations following diarrheal insult. Children who were born stunted and children weaned before two years of age exhibited more profound reductions in microbial metrics than the broader population. Our group recently showed that diversity acquisition was slowed among children born stunted, and that this was exacerbated among those children receiving no breastmilk; these findings further illustrate the apparent microbial vulnerability to insult observed among this group. These observations together may illustrate a possible mechanism through which children with intrauterine growth restrictions experience long term immunological and growth deficits, and merits further research. Further, it underscores the need to reinforce existing recommendations regarding continued breastfeeding to two years, rather than focusing only on exclusive breastfeeding in the first 6 months of life.

In contrast to the prior literature, our study was able to leverage longitudinal data and additionally detect associations between compromised microbial metrics and subsequent diarrheal incidence, lending further weight to the hypothesis that perturbations to gut microbiota may be a factor in driving the cyclical pattern of repeated diarrheal disease in early life. The majority of evidence demonstrating the association between gut perturbations and susceptibility to diarrheal disease has been derived from describing opportunistic *C. difficile* infections thriving in the diminished gut community following antibiotic treatment. Recent evidence has also implicated the presence and abundance of particular taxa in facilitating *Campylobacter* colonization (57–60). Here, we provide evidence that reduced microbial diversity is associated with increased subsequent frequency of all-cause diarrhea, after adjustment for other key predictors of illness such as breastfeeding intensity and category, asymptomatic pathogen pressure, dietary diversity and anthropometric status. While this does not denote causality, our study is able to illustrate temporal trends that have not been demonstrated before.

Taken together, this illustrates a growing body of evidence implicating disruptions to the gut microbiota as a potential mechanism through which diarrheal disease enacts long-term consequences for child health. Other studies described have also found significant associations between diarrheal disease and relative abundance of particular taxa, and highlighted the role of specific pathogens as predictors of microbial outcomes, providing impetus to conduct further analyses within the extensive MAL-ED data repository, in Peru and other sites, to characterize these relationships in more detail. Disaggregating exposures by pathogen and also by kind of diarrhea, ie, invasive or inflammatory, will also serve to further clarify the observed findings. Given the failure of water, sanitation and hygiene interventions to reduce diarrheal disease transmission and improve child growth, this is an important avenue for future research that may yield novel therapeutic techniques to interrupt the cycle of repeated diarrheal disease among young children worldwide.

Table 1: Diarrhea, growth, breastfeeding practices and gut microbial indices at each quarter of life in the Santa Clara birth cohort, Peru (n=271)

	Mean (SE) or % (n); *95% CI*			
Diarrheal disease exposures	6m	12m	18m	24m
No. pathogens per diarrheal sample	0.8 (0.1) *0.7, 0.9*	1.3 (0.1) *1.2, 1.1*	1.5 (0.1) *1.4, 1.6*	1.6 (0.0) *1.5, 1.7*
No. distinct diarrheal episodes	2.0 (0.1) *1.7, 2.2*	4.5 (0.2) *4.1, 4.9*	7.1 (0.3) *6.5, 7.7*	8.8 (0.4) *8.0, 9.5*
Duration of diarrheal episodes	4.7 (0.2) *4.2, 5.1*	4.2 (0.1) *4.0, 4.5*	3.9 (0.1) *3.7, 4.1*	3.7 (0.1) *3.5, 3.9*
Diarrheal severity score (CODA)	1.8 (0.1) *1.7, 2.0*	2.2 (0.1) *2.0, 2.4*	2.3 (0.1) *2.2, 2.5*	2.2 (0.1) *2.1, 2.3*
% diarrheal stools with at least one pathogen detected	56.5 (2.6) *51.3, 61.7*	71.7 (1.8) *68.2, 75.4*	78.0 (1.4) *75.1, 80.8*	80.5 (1.3) *78.0, 83.0*
Linear growth (LAZ)				
Mean length-for-age Z-score	-1.3 (0.1) *-1.4, -1.2*	-1.6 (0.1) *-1.7, -1.5*	-1.8 (0.1) *-2.0, -1.7*	-1.9 (0.1) *-2.0, -1.8*
% (n) stunted (LAZ<-2)	22.2 (60) *17.2, 27.2*	31.5 (78) *25.6, 37.3*	42.9 (96) *36.3, 49.4*	40.1 (87) *34.2, 47.5*
% (n) severely stunted (LAZ<-3)	4.1 (11) *1.7, 6.4*	5.3 (13) *2.5, 8.0*	10.3 (23) *6.3, 14.3*	10.8 (23) *6.6, 15.0*
Breastfeeding categories				
% (n) exclusively breastfed	0	0	0	0
% (n) predominantly breastfed	3 (8)	0	0	0
% (n) partially breastfed	96 (254)	94 (228)	52 (114)	16 (32)
% (n) weaned	1 (2)	5 (13)	48 (107)	84 (173)
Microbial diversity indices				
Shannon Diversity	1.5 (0.1) *1.4, 1.6*	2.8 (0.1) *2.7, 2.9*	3.9 (0.1) *3.7, 4.0*	4.6 (0.1) *4.5, 4.8*
CHAO1	79.5 (2.2) *75.1, 83.8*	132.0 (3.1) *125.9, 138.0*	191.4 (3.8) *183.9, 198.9*	237.3 (3.8) *22.7, 244.9*
Phylogenetic diversity	6.0 (0.2) *5.7, 6.3*	10.3 (0.2) *9.9, 10.8*	14.1 (0.3) *13.6, 14.7*	17.6 (0.3) *17.1, 18.1*
Simpsons Diversity[¥]	0.4 (<0.1) *0.4, 0.4*	0.7 (<0.1) *0.7, 0.7*	0.8 (<0.1) *0.8, 0.8*	0.9 (<0.1) *0.9, 0.9*

Table 2: Associations between diarrheal disease and gut diversity and richness outcomes in the Santa Clara cohort (n=271)

	Mean change in Microbial Metrics			
	Shannon	CHAO1	Phylogenetic Diversity	Simpson
	β (SE); *95% Confidence interval*			
Number of diarrheal episodes	-0.024 (0.008) *-0.040, -0.008*	-1.348 (0.450) *-2.230, -0.466*	-0.089 (0.032) *-0.152, -0.027*	-0.003 (0.002) *-0.006, -0.001*
Mean duration of diarrheal episodes (days)	-0.042 (0.018) *-0.069, -0.015*	-2.050 (0.785) *-3.587, -0.510*	-0.139 (0.055) *-0.246, -0.031*	-0.006 (0.003) *-0.011, -0.002*
Mean severity of diarrheal episodes (CODA points)	-0.072 (0.024) *-0.120, -0.025*	-3.609 (1.387) *-6.328, -0.891*	-0.252 (0.097) *-0.443, -0.061*	-0.007 (0.004) *-0.015, 0.002*
Time since last diarrheal episode (months)	0.022 (0.010) *0.002, 0.042*	1.028 (0.583) *-0.115, 2.170*	0.107 (0.040) *0.028, 0.186*	0.003 (0.002) -0.001, 0.007

All associations were adjusted for age, breastfeeding intensity and category, dietary diversity, antibiotic exposure, pathogen pressure. Time since last episode was additionally adjusted for year of life. Findings significant at the p<0.05 threshold are shown in green.

Table 3: Associations between diarrheal disease and microbial indices in sub-cohort of children with lagged diarrhea and antibiotic exposure (n=71)

	Mean change in Microbial Metrics			
	Shannon	CHAO1	Phylogenetic Diversity	Simpson
	β (SE); *95% Confidence interval*			
Number of diarrheal episodes	-0.047 (0.020) *-0.087, -0.008*	-1.451 (1.236) *-3.872, 0.971*	-0.156 (0.085) *-0.323, 0.010*	-0.006 (0.004) *-0.014, 0.001*
Mean duration of diarrheal episodes (days)	-0.042 (0.018) *-0.069, -0.015*	-4.032 (2.027) *-8.010, -0.059*	-0.319 (0.140) *-0.594, -0.045*	-0.010 (0.005) *-0.021, 0.000*
Mean severity of diarrheal episodes (CODA points)	-0.096 (0.048) *-0.190, -0.001*	-4.973 (3.092) *-11.032, 1.087*	-0.298 (0.164) *-0.717, 0.121*	-0.011 (0.008) *-0.027, 0.005*

All associations were adjusted for age, breastfeeding intensity and category, dietary diversity, antibiotic exposure, pathogen pressure. Findings significant at the p<0.05 threshold are shown in green.

Table 4: Associations between microbial indices and subsequent diarrheal episodes in the Santa Clara cohort (n=271)

	Shannon	CHAO1	Phylogenetic Diversity	Simpson
	β (SE); *95% Confidence interval*			
After 6m of age	-1.25 (0.45) *-2.13, 0.36*	-0.01 (0.01) *-0.03, 0.01*	-0.15 (0.16) *-0.46, 0.17*	-3.36 (1.52) *-6.35, -0.38*
After 12m of age	-0.53 (0.23) *-0.98, -0.09*	-0.01 (0.01) *-0.02, -0.01*	-0.16 (0.07) *-0.29, -0.03*	-1.76 (1.18) *-4.08, 0.56*
After 18m of age	-0.17 (0.15) *-0.45, 0.12*	-0.01 (<0.01) *-0.01, 0.00*	-0.01 (0.03) *-0.08, 0.07*	-1.81 (0.99) *-3.75, 0.13*

Coefficients represent mean change in the number of subsequent diarrheal episodes experienced accompanying a 1-point increase in each microbial metric. All associations were adjusted for breastfeeding intensity and category, dietary diversity, antibiotic exposure, pathogen pressure, age and LAZ at birth. Findings significant at the p<0.05 threshold are shown in orange.

Supplementary Table 1: Diarrhea, growth, breastfeeding practices and gut microbial indices at each quarter of life in sub-cohort for analysis of lagged exposures (n=71)

	Mean or % (SE); ***95% CI***			
Diarrheal disease exposures	**6m**	**12m**	**18m**	**24m**
No. pathogens per diarrheal sample	0.7 (0.1) *0.5, 0.8*	1.4 (0.1) *1.2, 1.6*	1.6 (0.1) *1.5, 1.8*	1.7 (0.1) *1.6, 1.9*
No. distinct diarrheal episodes	1.0 (0.1) *0.7, 1.2*	2.6 (0.3) *2.1, 3.2*	4.5 (0.3) *3.8, 5.2*	5.4 (0.4) *4.6, 6.1*
Duration of diarrheal episodes	4.1 (0.3) *3.5, 4.8*	4.0 (0.2) *3.5, 4.4*	3.6 (0.2) *3.2, 3.9*	3.4 (0.2) *3.1, 3.7*
Diarrheal severity score (CODA)	1.6 (0.2) *1.2, 1.9*	2.0 (0.2) *1.7, 2.3*	2.2 (0.1) *1.9, 2.4*	2.0 (0.1) *1.8, 2.2*
% diarrheal stools with pathogen detected	52.5 (6.6) *39.2, 65.9*	75.9 (3.8) *68.2, 83.6*	83.4 (2.3) *78.8, 87.9*	85.8 (1.8) *82.1, 89.4*
Linear growth (LAZ)				
Mean length-for-age Z-score	-1.4 (0.1) *-1.7, -1.2*	-1.6 (0.1) *-1.8, -1.4*	-1.9 (0.1) *-2.2, -1.7*	-2.0 (0.1) *-2.2, -1.8*
% (n) stunted (LAZ<-2)	26.7 (19) *16.2, 37.3*	31.0 (22) *20.0, 42.0*	43.7 (31) *31.8, 55.5*	45.1 (32) *33.2, 56.9*
% (n) severely stunted (LAZ<-3)	9.9 (7) *2.8, 17.0*	8.5 (6) *1.8, 15.1*	11.3 (8) *3.7, 18.8*	8.5 (6) *1.8, 15.1*
Breastfeeding categories				
% (n) exclusively breastfed	0	0	0	0
% (n) predominantly breastfed	4 (3)	0	0	0
% (n) partially breastfed	96 (68)	96 (68)	44 (31)	13 (9)
% (n) weaned	0	4 (3)	56 (40)	87 (62)
Microbial diversity indices				
Shannon Diversity	1.74 (0.09) *1.56, 1.93*	2.85 (0.10) *2.66, 3.04*	3.90 (0.12) *3.65*	4.64 (0.09) *4.46, 4.82*
CHAO1	98.9 (4.9) *89.1, 108.7*	141.8 (5.0) *131.8, 151.7*	202.7 (6.8) *189.0, 216.3*	236.9 (6.6) *223.7, 250.0*
Phylogenetic diversity	6.9 (0.3) *6.4, 7.6*	10.7 (0.3) *10.1, 11.3*	14.6 (0.5) *13.6, 15.5*	17.2 (0.4) *16.3, 18.0*
Simpsons Diversity	0.4 (<0.1) *0.4, 0.5*	0.7 (<0.1) *0.6, 0.7*	0.8 (<0.1) *0.8, 0.9*	0.9 (<0.1) *0.9, 0.9*

Figure 1: Time to first diarrheal episode (a) and proportion experiencing diarrhea at each month of life (b) in the Peru Microbiota Collaboration

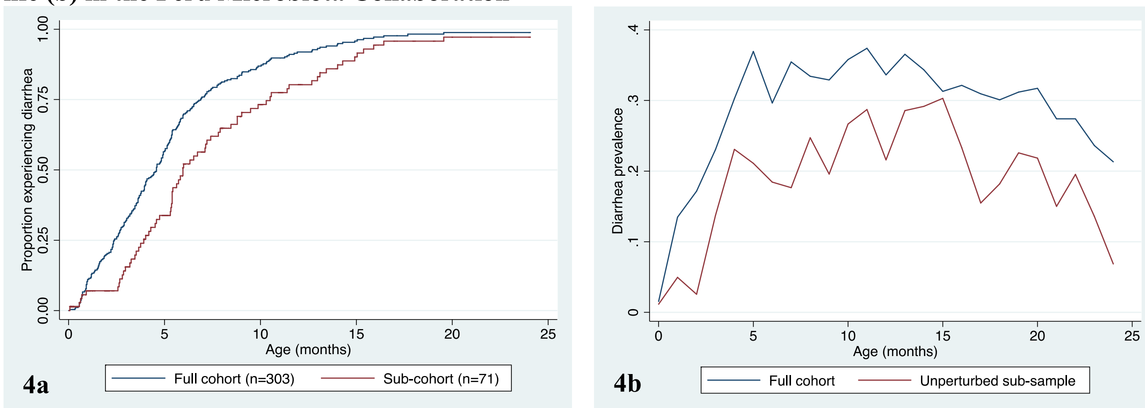

Figure 2: Diversity and richness indices increase with age in the Peru Microbiota Collaboration (n=271)

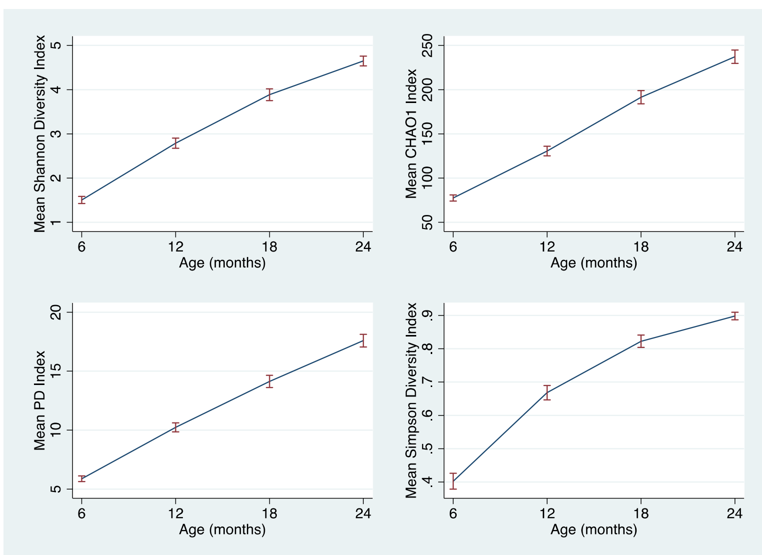

Figure 3: Frequency, duration and severity of diarrheal episodes associated with reduced diversity and richness, and time since diarrhea is associated with recovery of microbial metrics among children in Peru

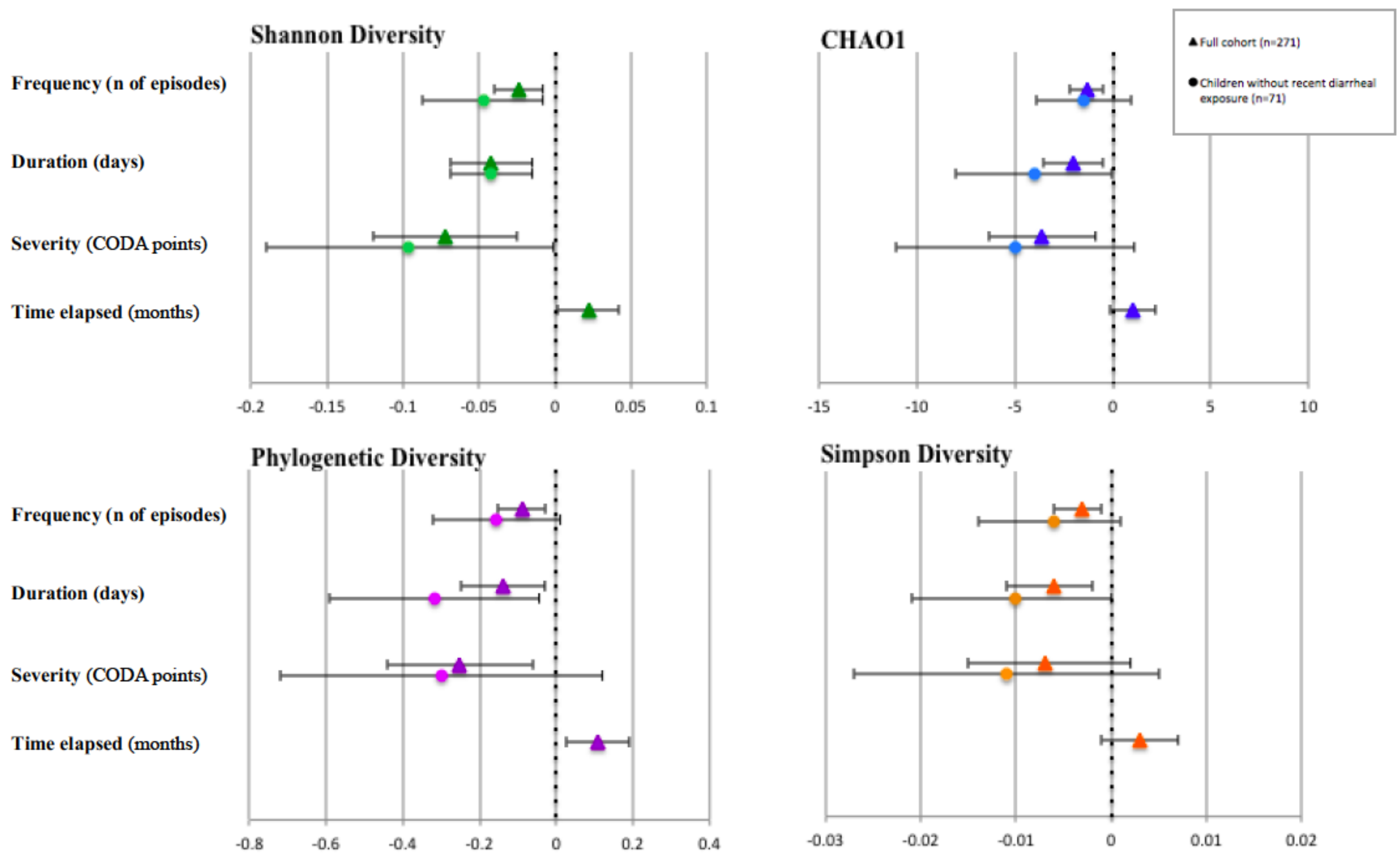

Figure 3 shows regression (β) coefficients showing the effect and direction of association between diarrheal episodes, duration, severity among 271 children in the full cohort (Δ) and 71 children whose samples were contributed after at least one month without diarrheal symptoms (O). We further demonstrate positive associations between time elapsed since last episode and microbial diversity and richness in the full cohort. All models were adjusted for age, breastfeeding intensity and category, dietary diversity, antibiotic exposure, pathogen pressure; Time since last episode was additionally adjusted for year of life.

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Chapter 6: Gut microbial members indicative of Campylobacter burden and linear growth shortfalls in a peri-urban community in Peru

ABSTRACT.

Background. Interactions between enteric infections and undernutrition continue to drive mortality and morbidity among children living in poverty. Infection with enteropathogens like *Campylobacter* spp. results in growth impairment and other long-term disabilities even in the absence of symptoms, and it has been posited that changes incurred to the gut microbiota during or after infection may in part explain these sequelae. No studies have specifically assessed the potential changes to the gut environment incurred by *Campylobacter* infections among young children in poverty, who suffer the undue burden of the cycle between undernutrition and infection. We evaluate associations between *Campylobacter* infections, linear growth, and features of the fecal microbial community in a birth cohort of 271 children living in Amazonian Peru.

Methods. Routine fecal samples at 6, 12, 18 and 24m of age (n=928) were used to identify gut bacterial taxa by polymerase chain reaction (PCR) detecting 16S ribosomal RNA, and generate metrics of diversity and richness (Shannon, Simpson, CHAO1 and Phylogenetic diversity). Asymptomatic surveillance stools were collected monthly from 0-12 month and quarterly thereafter for identification of *Campylobacter* using enzyme-linked immunoassays (ELISA). Diarrheal samples were ascertained during each reported episode from 0-24m of age.

Monthly anthropometric measurements were collected to calculate standardized length-for-age Z-scores (LAZ). Associations between symptomatic and asymptomatic

Campylobacter infections, community and species-level microbial metrics, and child growth were investigated using multiple linear regression with adjustment for within-child correlations, age, breastfeeding and infection with other pathogens. Indicator species analysis was employed to identify possible gut taxa associated with *Campylobacter* infections.

Results. By two years of age, 251 (93%) of all children in the cohort had *Campylobacter* present in asymptomatic stools, and 221 (82%) experienced infection with diarrhea. Asymptomatic infection was associated with reductions in length-for-age concurrently and at 3, 6, and 9m thereafter (β=0.02, p<0.01 across all time points). Each additional episode of *Campylobacter*-diarrhea was associated with a concurrent reduction in -0.03 LAZ (p=0.002), independently from all-cause diarrhea. Asymptomatic *Campylobacter* infections were associated with changes to the gut microbial environment. Infection was associated with increased microbial diversity and richness. Indicator species analysis identified thirteen taxa indicative of being in the highest quartile of infection from birth to two years of age, three of whose presence showed evidence of association with lower concurrent LAZ. Nine additional indicators of low *Campylobacter* burden were identified, the majority of which were butyrate-producing *Firmicutes.* Presence or abundance of four taxa indicative of low infection burden was associated with higher LAZ concurrently and one month thereafter.

Conclusions. *Campyobacter spp* infections were nearly universal among children in the Peruvian Amazon in the first two years of life. We illustrate changes to gut microbial populations associated with asymptomatic infection in a longitudinal study with a large sample size and generate testable hypotheses for future research to guide interventions

related to the improved control of *Campylobacter* and mitigation of its adverse effects on growth in infancy and early childhood.

INTRODUCTION.

Interactions between enteric infections and undernutrition and their toll on quality of life and survival of young children in lower and middle-income countries (LMIC) have been extensively described over the last several decades (1,2,11,3–10). Nonetheless, childhood stunting, or chronic undernutrition, continues to affect over 160 million children annually and underlie a vast proportion of child deaths worldwide (12,13). The aggregate effect of interventions aimed at improving child growth has been disappointing, and it is widely accepted that understanding of the mechanisms driving these interactions remains incomplete (14). This has led to increased interest in research specifically elucidating pathways through which enteric infections may drive stunting and its long-term consequences for vulnerable children worldwide in order to guide more effective interventions. An altered intestinal state known as *environmental enteropathy* (EE), characterized by intestinal and systemic inflammation and increased gut permeability, is proposed to play an important role in propagating the cycle of enteric infections and undernutrition (15–17). In recent years, a growing body of evidence has linked physiologic features of gut immune activation and permeability measures to long-term growth deficits and blunted immune responses, including vaccine failure (18–22). That EE can arise from chronic or repeated infections in the absence of diarrheal symptoms may help to explain persisting linear growth failure in settings where programs to prevent and treat clinical diarrhea and provide nutritional supplementation are well-implemented (13,15).

Infection with *Campylobacter* spp. has been suggested to illustrate the EE hypothesis for multiple reasons. In high-income settings where infection is the leading cause of bacterial gastroenteritis (23–25), there is extensive literature detailing long-term sequealae of disease. Studies linking infection to chronic conditions like Guillan-Barré syndrome, reactive arthritis, inflammatory bowel disease and irritable bowel syndrome have demonstrated that *Campylobacter* infections can impact immunological tolerance and gut permeability and function well beyond the cessation of acute symptoms (26–34). While data in LMIC is more limited, recent work has shown that symptomatic and asymptomatic infection are independently associated with linear growth acquisition in the subsequent three-month period, and that children with high burden are significantly shorter at two years of age (35,36). Observational and more recently metagenomic analysis confirms that prevalence of *Campylobacter* infections and presence of Campylobacteraceae in the gut are significantly increased in undernourished children (37–39). These observations are even more relevant in light of recent estimates of higher asymptomatic carriage and attributable fraction of diarrhea to *Campylobacter spp.*, and a wider range of species with clinical outcomes than previously appreciated (36,40–42). Indeed, the first large multi-site study to employ culture-independent diagnostic methodology to enumerate this burden found *Campylobacter* to have the highest bacterial attributable fraction of diarrhea, a high prevalence of asymptomatic carriage and significant associations between infection, increased systemic inflammation and increased intestinal permeability (36,40,42).

Together these findings have led to the suggestion that *Campylobacter* may be an illustrative example of how enteric infections can impair growth through the mechanism of EE (36). However, a more complete description of how enteropathogens may drive the EE phenotype, particularly when they occur in the absence of clinical symptoms is needed. One putative pathway is through enacting changes to the microbial ecology of the gut, which in turn may disrupt the integrity and functioning of intestinal immunity and nutrient harvest. Extensive animal and in vitro studies have illustrated the role of gut microbiota in maintaining healthy immune responses and gut barrier integrity (43–46), key features altered in EE, and experimental studies show improved immunogenicity to enteric vaccines after exposure to different pre- and probiotic strains in mice (47–49). Gut microbiota are also known to affect nutrient and energy harvest (50–53) and are increasingly implicated in child growth. Induction and reversal of weight gain can be achieved by inoculating germ free mice with fecal specimens from children of different growth profiles (52,54–56), and human studies have found differences in presence and abundance of specific phyla and reductions in overall bacterial diversity among children with severe acute malnutrition (54,57–59).

It is therefore plausible that consistent exposure to enteropathogens disrupts childhood immunity and growth in part through the alteration of gut microbiota. Indeed, studies have shown that diarrhea can have lasting impacts on gut microbial populations (60–64), though most have enrolled adults in high-income settings. Two large-scale studies of children in LMIC settings have recently suggested that taxonomic composition and microbial diversity were significantly compromised during and after diarrheal disease (65), that these changes were exacerbated by severity and duration of symptoms and can endure beyond one month after clinical disease. In the case of *Clostridium difficile* infection, researchers observed that asymptomatic carriers also had significantly reduced microbial diversity relative to healthy patients (66); however, this study enrolled fewer than 10 individuals per arm, whose ages ranged from 25 to 86 and whose comorbidities were not matched. No other studies specifically evaluating impacts of asymptomatic enteric infection on gut microbial members or assembly are available to our knowledge.

The evidence described supports the hypothesis that gut microbiota are an important factor in determining the complex interplay between infection, diarrhea, immunological responses and child growth, but to advance this theory key gaps in research must be addressed. A more complete description of the possible impacts of clinical *and* subclinical infections on the gut microbial community are needed, followed by an evaluation of whether these potential changes are implicated in key inflammatory or metabolic pathways that may drive long-term health deficits. The current study proposes to explore these questions using data from a community-based longitudinal birth cohort in Amazonian Peru. We describe the magnitude and direction of associations between *Campylobacter* infection and diarrhea, linear growth, and fecal microbial diversity and composition with an aim to contribute to evidence of the putative pathways between intestinal infection and growth failure in early childhood.

METHODS.

Study design & setting. This study was nested within the Etiology, Risk Factors, and Interactions of Enteric Infection and Malnutrition and the Consequences for Child Health and Development (MAL-ED) Study, a longitudinal study based in eight settings characterized by high childhood undernutrition and diarrhea (67). The Peru Microbiota Collaboration was formed between Asociación Benéfica PRISMA in Iquitos, Peru, the Johns Hopkins Bloomberg School of Public Health in Baltimore, MD, and the Gordon Laboratory at Washington University in St. Louis, MO in 2016 to characterize the role of the infant gut microbiota in interactions between enteric infections and nutritional indicators that MAL-ED was enacted to explore. Detailed methodology of the collection of demographic and demographic data, biological specimens and their analysis is therefore available in the extensive literature published by the MAL-ED Consortium (15,67–71). In brief, the research site from which data was ascertained for this study is located in a peri-urban community in the Amazonian lowlands of Iquitos, Peru, which is marked by low access to water and sanitation and high incidence of diarrheal disease and childhood stunting. Three-hundred and three children across three local communities were enrolled within 17 days of birth and followed to two years of age between November 2009 and February 2012. These children contributed routine stool samples for surveillance of asymptomatic enteric infections at monthly intervals during the first year of life, and quarterly intervals thereafter (15, 18, 21 and 24m of age). Weight and length were also measured monthly for the calculation of nutritional growth indices. Community members trained as fieldworkers visited the homes of all participants twice weekly to record illness and treatment (antibiotic) history, breastfeeding behaviors and dietary intake. In the case of reported diarrheal illness, additional stool samples were taken for the diagnosis of clinical disease. Routine stools collected at 6, 12, 18 and 24m of age were retrospectively selected for inclusion in the Peru Microbiota Collaboration and sent to the Gordon Laboratory in St. Louis.

Diagnostics and microbial analysis. Upon initial collection, stool samples were placed into Cary-Blair transport media within two hours of production and processed in the satellite laboratory in Iquitos the same day. *Campylobacter* were identified in fecal samples using a ProSpecT *Campylobacter* enzyme-linked immunosorbent assay (ELISA). While culture methodology was also available and carries the advantage of species differentiation, the MAL-ED study was the first to reveal the considerably increased burden of *Campylobacter* using culture-independent methods. Details of diagnostic assays for other enteropathogens are detailed and published elsewhere (71). Stools selected for inclusion in the Peru Microbiota Collaboration were additionally processed at Washington University in St. Louis, Missouri for analysis of the gut microbiota. DNA was isolated from aliquots of pulverized, frozen fecal samples. Fecal samples were homogenized by bead beating for 4 minutes (MiniBeadbeater-96™, Biospec Products; Bartlesville, Oklahoma USA) in a mixture of 250 µL of 0.1 mm-diameter zirconia/silica beads (and a 3.97 mm-diameter steel ball for a subset of samples), 710 µL of 500:210 2X buffer A (200 mM NaCl, 200 mM Tris, 20 mM EDTA):20%SDS, and 500 µL of 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. Samples were then centrifuged at 3,220 x g for 4 min at room temperature. 350 uL of aqueous phase was transferred to a new tube containing 500 uL 25:24:1 phenol:chloroform:isoamyl alcohol, pH-adjusted to alkaline. The tubes were inverted 10 times to mix and then centrifuged at 3220g X 4 min. 250 uL of aqueous phase was transferred to a 96-well plate. DNA was purified from a 100 uL aliquot of each 250 uL crude DNA sample by mixing 100 uL of crude DNA with 400 uL of a 675:45 mix of Qiagen® buffer PM:3M NaOAc, pH5.5 and then passing the mixture through a Qiagen® QIAquick96 plate by centrifugation at 3220g X at least 4 min. Wells were washed twice by passing 900 uL Qiagen® buffer PE through the plate by centrifugation at 3220g X at least 2 min. Quant-iT dsDNA Broad-Range Assay Kits (Invitrogen; Carlsbad, California, USA) was used to quantify the pure DNA.

For each DNA sample, the V4 regions of bacterial 16S rRNA (V4-16S rRNA) genes present were amplified using previously published primers (59) and the following PCR conditions: an initial denaturation step of 2 min at 94°C, followed by 26 cycles of 15 sec at 94°C, 30 sec at 50°C, and 30 sec at 68°C, followed by a final step at 68°C for 2 min. Amplicons were sequenced using an Illumina MiSeq instrument. The resulting 250-nucleotide paired-end reads were trimmed to 200 bases and merged with Flash, and QIIME (v 1.9.0) (72) was used to demultiplex the reads and cluster them into operational taxonomic units (OTU; clusters sharing ≥97% sequence identity). OTU that failed alignment by PyNast (73) were removed from the dataset. Taxonomy was assigned to each OTU using the Ribosomal Database Project (RDP) classifier version 2.4 (74) and the 2013 Greengenes reference database (75). The resulting OTU table contained 32,300±16,453 (mean±sd) reads per sample. Subsequently, OTU that did not have relative abundances of ≥1% in at least one sample or ≥0.1% of the reads in at least two samples were removed from the data. The data were then rarefied to 11,000 reads per sample, and Shannon's diversity index, the total phylogenetic diversity, and the observed number of OTU for each community were calculated as measures of microbial richness and diversity. Samples that did not have at least 11,000 reads were removed from the data.

Analytic approach.

Definition of covariates. Diarrhea was defined as ≥3 loose stools in a 24-hour period, with distinct episodes separated by either a pathogen negative stool or a period of ≥14 days without symptoms. Diarrheal severity was measured using a community diarrheal assessment tool (76) classifying severity as a function of maternally reported presence and duration of fever, vomiting, anorexia, liquid stools, and the maximum number of stools in any 24-hour period during the episode. Detection of enteropathogens in surveillance stools in the absence of diarrheal symptoms was considered asymptomatic infection. ‘Pathogen pressure’ was defined as an aggregate measure denoting asymptomatic infections and was calculated as mean number of enteropathogens per surveillance stool contributed. Breastfeeding was considered using a categorical metric denoting whether a child was currently being exclusively, predominantly, or partially breastfed or weaned, according to WHO definitions (77). The number of WHO food groups (0-7) that a child had ever ingested at each time of follow-up was included as a measure of dietary diversity (77). Standardized length-for-age Z-scores (LAZ) were calculated using the World Health Organization’s Multicentre Growth Reference Study Group guidelines, with children measuring at below -2 LAZ classified as stunted (78).

Campylobacter diarrhea was considered as a continuous variable denoting the number of distinct *Campylobacter*-positive diarrheal episodes experienced in a child’s life at given time point. Asymptomatic *Campylobacter* infection was assessed using an aggregate, time-varying variable expressing the proportion of all surveillance stools contributed by a single child with *Campylobacter* detection. Using this proportion, we also calculated quartiles of asymptomatic *Campylobacter* burden at each quarter of life. We defined persistent asymptomatic infection as three consecutive monthly infections in the first year of life; we were unable to estimate the burden of persistent infections in the second year of life due to absence of monthly measures.

We assessed community-level indicators of the infant gut microbiota using four metrics of within-sample (alpha) diversity and richness. The Shannon Diversity Index (SDI) (79) accounts for the number and distribution of different species, and Simpson's Diversity (80) describes the sum of the squared frequency of all OTU within a given sample. Bacterial richness was measured by the CHAO1 metric(81), which uses a capture-recapture method to enumerate species richness, and by taking the sum of all phylogenetic branch lengths represented within a sample as a measure of phylogenetic diversity (82). To explore changes to individual members of the gut microbial community, we modeled presence and abundance of individual OTU as outcomes of infection and as predictors of linear growth.

Statistical analyses. Descriptive analysis of *Campylobacter* infection and diarrhea was conducted using Kaplan Meier curves and survival analysis allowing for multiple failures per child. Associations between infection, diarrhea, and child growth were measured using multiple linear regression (MLR) with a generalized estimating equations (GEE) approach to adjust for within-child clustering. Growth models were adjusted for breastfeeding, dietary diversity, diarrhea, pathogen pressure and antibiotic exposure (number of days with any exposure). Models with concurrent LAZ as the outcome were adjusted for LAZ at birth; analyses modeling subsequent LAZ (at 1, 3, 6, 9m thereafter) were adjusted for LAZ at current time point.

We employed the same methodology (MLR with GEE) to describe associations between *Campylobacter* infection and diarrhea on metrics of microbial diversity and richness from birth to two years of age. Microbial diversity and richness models were adjusted for age as well as metrics of breastfeeding, diet and antibiotic use defined above.

To ascertain whether *Campylobacter* had an independent effect on these metrics, models with asymptomatic infection and diarrheal episodes as predictors were additionally adjusted for proportion of surveillance stools with *any* enteropathogens and number of all-cause diarrheal episodes, respectively.

To assess relationships between *Campylobacter* exposure and specific fecal OTU, we conducted Indicator Species Analysis (ISA) comparing children in different categories of asymptomatic infection and diarrhea burden. ISA (83) is a statistical procedure identifying particular OTU that are significantly *indicative* of a particular group of samples identified by a priori characteristic of interest. Indicator species values are calculated for every OTU for each group of communities, as the product of the proportion of communities in a group in which the OTU is detected and the mean abundance of the OTU in that groups, normalized to the sum of mean abundances in all groups. The values generated range between 0 and 1, with a perfect indicator species occurring only in one group. Very common conserved species that occur in all groups are poor indicators, as are very rare species that are not sampled often. Hypothesis tests are performed by permutations in which samples are randomly assigned to groups to generate a distribution of values; the null hypothesis is supported if the distribution of values does not differ in the test case when compared to those generated though permutations (83). OTU not found in at least 20% of the children in at least one of the *Campylobacter* infection groups were excluded from the analysis.

ISA was conducted to identify possible OTU denoting recent *Campylobacter*-positive diarrheal history comparing the following groups: *Campylobacter* – positive versus negative diarrheal stools; stools contributed by children with versus without diarrhea in the last 7 days; and stools contributed by children with versus without diarrhea in the last 14 days. Iterations of ISA were conducted at each time point to assess asymptomatic *Campylobacter* history: comparison of *Campylobacter* – positive versus negative surveillance stools, and comparison of stools contributed by children in the highest versus lowest quartile of asympomatic *Campylobacter* burden. We replicated these analyses using groups defined by exposure of the next three most prevalent enteropathogens in the cohort (*Giardia spp.,* entero-aggregative *Escherichia coli* (EAEC), and norovirus) in order to provide a platform for comparison of the number and nature of indicator species that may be identified in this cohort. Adjusted p-values were derived using a Benjamini-Hochberg method to correct for false discovery rates arising from multiple comparisons (84).

To advance our enquiry as whether modification of gut microbes by *Campylobacter* infection may be implicated in the observed relationships between *Campylobacter* and child growth, we explored whether outputs of the ISA were independently associated with LAZ scores and stunting from birth to two years of age. We built MLR models using individual OTU identified as indicative of *Campylobacter* burden as predictors of concurrent and future (+1m) LAZ and stunting in a model adjusting for age, LAZ at birth, breastfeeding, antibiotic exposure, dietary diversity, pathogen pressure and diarrheal disease.

RESULTS.

Campylobacter infection and diarrhea in the population. Two-hundred and seventy-one (89.4%) of the 303 children were included in the Peru Microbiota Collaboration; the remaining 32 children who were not included were lost to follow-up prior to 6m of age. In total, 146 boys and 125 girls contributed 6096 months of person-time under follow-up, 6011 surveillance stools and 2440 diarrheal samples to the study. Using ELISA as the gold standard, we observed only 22% (364) of *Campylobacter*-positive stools yielded an isolate identified as Campylobacter by culture methods, and this proportion was unchanged when we stratified by specimen type (22% in both diarrheal samples and surveillance stools). *Campylobacter jejuni,* or hippurate positive isolates, accounted for half of all culture-positive specimens (48%), and observation was again consistent across surveillance and diarrheal samples alike (45% of diarrheal samples and 48% of surveillance stools).

Campylobacter infections were common and often persistent. *Campylobacter spp* was detected in approximately one-fifth of all surveillance stools tested (22%, 912 samples). By two years of age, 251 (93%) of all children in the cohort experienced at least one *Campylobacter* detection in the absence of symptoms. Mean time to first infection was 7.8 months, and the majority of children (64%, 169 children) were *Campylobacter*-positive in surveillance stools before ever presenting with *Campylobacter*-positive diarrhea. Table 1 summarizes *Campylobacter* infection and diarrhea at each quarter of life from birth to two years of age. Infection was more prevalent in the second year of life, though the odds of detection in the first year increased significantly with each additional month of age (OR 1.21, $p<0.001$). The burden of infection was skewed across the cohort; mean number of infections among 251 children was 3.4, but 44 children experienced >5 infections and 5 children experienced >10 infections before their 2nd birthday. We additionally observed that 55 children

(20.3%) experienced persistent *Campylobacter* infections in the first year of life. Sixty-nine (8%) of the 868 *Campylobacter*-positive surveillance samples were collected while children were being treated with any antibiotics, with 2% (21 samples) specifically collected during concurrent macrolide exposure.

Of the 260 children who experienced diarrhea in the cohort, 221 (85%) contributed at least one specimen in which *Campylobacter* was detected. In total, *Campylobacter* was detected in 31% (758) of samples taken during reports of diarrhea in the first two years of life. Mean age at first reported *Campylobacter*-positive diarrhea was 9.3 months. Children experienced an average of 2.6 episodes of *Campylobacter*-positive diarrhea by 2 years of age; however, this was highly variable between children, ranging from 0 – 15 detections. Approximately a quarter of episodes were treated with antibiotics (27.3%, 169 episodes) overall, with 15% (93 episodes) were treated with macrolides.

Campylobacter infections and linear growth. *Campylobacter* infection and diarrhea were associated with reduced child growth. After adjustment for breastfeeding frequency and status, diarrhea, antibiotic exposure, pathogen pressure, and LAZ at birth, children had, on average, a 0.01 lower LAZ score associated with a 10% increase in the proportion of asymptomatic stools with *Campylobacter* detected. When we repeated these analyses, adjusting for LAZ at current time point, a 10% increase in detections was associated with a 0.02 reduction in LAZ at 3, 6, and 9 months thereafter ($\beta=0.02$, $p<0.01$ across all time points). *Campylobacter*-positive diarrhea was associated with growth independently from all-cause diarrhea. In a model adjusting for number of diarrheal episodes, mean number of pathogens detected per surveillance stool, antibiotic exposure, and breastfeeding intensity and category each additional episode of *Campylobacter*-diarrhea was associated with a reduction in -0.03 LAZ (95% CI -0.04, -0.01; p=0.002). However, this was not found when we modeled impacts of episodes on future LAZ thereafter.

Associations between *Campylobacter* spp infection, diarrhea and gut microbial features.

Microbial richness and diversity. We did not observe metrics of microbial diversity and richness to be associated with lifetime history of *Campylobacter* infections or diarrhea independently from overall infection and disease history (Table 2). There was no evidence that *Campylobacter*-specific diarrheal severity or duration had an independent effect on these metrics, after accounting for diarrhea with other etiologies. Metrics of microbial diversity and richness were positively associated with *Campylobacter* detection in the absence of diarrhea, independently of overall asymptomatic pathogen pressure and frequency.

Indicator Species Analysis. We conducted indicator species analyses (ISA) at 6, 12, 18 and 24m of age to identify possible taxonomic units significantly associated with having a high burden of *Campylobacter* infection and diarrhea. At 6, 12 and 18m of age, *Campylobacter* spp was the only OTU that differed significantly between *Campylobacter* -positive and negative surveillance stools (p=0.005, p=0.07, p=0.028 respectively). However, at 24m of age, we did see a significant increase in a member of the families *Coriobacteriaceae* and *Ruminococcaceae*, both of which were significantly more abundant in and specific to *Campylobacter*-positive stools at 24m of age (p=0.02, p=0.03 respectively). When assessing lifetime *Campylobacter* burden as the risk factor, we identified 23 OTU that were significantly indicative being in extreme quartiles from birth to two years of age. Of these, 9 OTU were indicative of being in the low burden group at

6, 12 and 24m, and 14 OTU were indicative experiencing high burden at 6, 18 and 24m of age. Results of the ISA comparing children in the highest and lowest quartiles of asymptomatic *Campylobacter spp* infection are shown in Table 3. No OTU were identified as indicative of *Campylobacter* diarrhea history.

We replicated ISA analyses for three other highly prevalent enteropathogens in the cohort to assess whether results were indicative of *Campylobacter spp* burden specifically, or of general enteric pathogen pressure. Surveillance stools contributed by children of high and low *Giardia spp.,* entero-aggregative *Escherichia coli* (EAEC), and norovirus infection were compared at each quarter of life. At 6m of age, we identified *Streptococcus thermophiles* as indicative of being in the lowest-burden quartile of norovirus infection (p=0.02). Five OTU in total were significantly indicative of giardia burden throughout life; three at one year of age and two at 18m of age. No other OTU were identified as being indicative of EAEC, giardia or norovirus in the cohort.

Associations between gut microbial species and metrics of child growth. To further explore evidence for the hypothesis that changes to the gut microbiota may be implicated in the relationship between asymptomatic *Campylobacter* infection and child growth deficits, we assessed whether OTU indicative of *Campylobacter* burden were independently associated with length-for-age in this population. The presence and abundance of each OTU listed in Table 3 were modeled as predictors of growth longitudinally from birth to two years adjusting for age, LAZ at birth, pathogen pressure, diarrheal history, antibiotic exposure, breastfeeding and dietary diversity. Results of longitudinal multivariable regression models measuring associations between the presence and abundance of each OTU derived from the ISA with concurrent and subsequent growth (1m after stool collection) metrics from birth to two years are shown in Tables 4 and 5.

While the presence of *Bifidobacterium breve* was nearly ubiquitous in the population, we observed its abundance was significantly associated with increased LAZ concurrently and one month later in the fully adjusted model. Presence of *B. breve* was also associated with reduced odds of being stunted one month after stool analysis; a 10% decrease in the odds of stunting was associated with each additional thousand units of *B. breve* detected throughout follow-up. We observed significant evidence that the presence of *Erysipelatoclostriudium ramosum* and *Ruminococcus torques*, which were found to be significantly indicative of low *Campylobacter* burden at two years of age, was positively associated with LAZ from birth to two years of age. In addition, abundance of *E. ramosum* was associated with increased LAZ at the time of follow-up, as well as 1m thereafter. *R. torques* was also significantly associated with the odds of stunting; children in whose stools this microbe was detected had a 37% reduction in the odds of being stunted at 6, 12, 18 and 24m of life ($\beta=0.63$; $p=0.03$). The presence of three microbes (*Parabacteroides distasonis*, *Faecalibacterium paruznitzii* and *Senegalimassilia anaerobia)* indicative of high *Campylobacter* burden at different stages of life was associated with significantly increased odds of being stunted at the following visit (OR 1.40, 1.43 and 1.58 respectively). However, we also observed increased odds of stunting associated with the presence of a member of the *Lachnospiraceae* family which was found to be indicative of low *Campylobacter* burden at 24m of age.

We further observed suggestive trends between the presence of additional OTU and child growth, for which the probability of alpha error was below 10% ($p<0.1$). Presence of *Streptococcus infantis,* which was indicative of being in the low-burden quartile of *Campylobacter* infection, was marginally associated with LAZ throughout life and was associated with a reduction in the odds of stunting at the following visit. Negative growth trends were also observed for *F. prauznitzii, S. dextrinosolvens, H. biformis* and a member of the *Prevotellaceae* family, all of which were indicative of the high burden quartile of *Campylobacter* infection (see Tables 4, 5).

DISCUSSION.

This study highlights a population experiencing extremely high incidence of *Campylobacter* infections and provides evidence for the hypothesis that asymptomatic burden is associated with changes to the gut microbial community, which in turn may impact child growth. In a longitudinal birth cohort of 271 children living in a peri-urban tropical environment with high exposure to enteropathogens, we identify several individual gut microbes that are indicative of experiencing heavy *Campylobacter* burden from birth to two years of age, and identify those whose presence or abundance is significantly associated with subsequent linear growth gains.

We additionally confirm previously observed relationships between clinical and subclinical *Campylobacter* infections and growth acquisition in children living in poverty in Peru (35,36).

Prior work within the Peru Microbiota collaboration (Chapter 5) has shown that the frequency, severity and duration of all-cause diarrhea is associated with lasting reductions in the acquisition of fecal microbial diversity. Here, we found no association between *Campylobacter* diarrhea with microbial diversity and richness indices, independent of all-cause diarrhea, nor did we identify key species indicative of recent disease. It is possible that symptoms accompanying clinical diarrhea affect the overall population diversity and richness indiscriminately; or if particular pathogens do enact a specific effects on the gut community, that *Campylobacter spp* are not among them. A recent study published results from ISA identifying numerous OTU associated with the diarrheal and recovery phases of *Vibrio cholerae* infection in Bangladesh; however, this was conducted in among hospitalized adults (85). Older individuals whose microbial ecosystem have reached a stable state may be more amenable to comparison using the ISA methodology, relative to infants whose gut environment is necessarily in flux during this critical developmental window. More evidence on impacts of diarrhea of various etiologies in community settings and among infants and young children will help to clarify these relationships. In addition, disaggregating all-cause diarrhea in future analyses to assess whether these results differ by pathogens inducing inflammatory or invasive disease is of interest to improving our understand of the consequences of diarrheal disease for the microbiota.

In contrast, we observed that asymptomatic *Campylobacter* infection is associated with increased alpha diversity among children age 0-2 years, independent of other enteropathogens. This finding highlights the caveats of judging gut microbial "health" based on diversity measures alone. In early life, increased diversity may not always be a marker of optimal health. Indeed, breastmilk has been shown to exert a suppressive effect on diversity in the infant gut by this group and others, leading to the hypothesis that limited diversity in early life reflects the healthy predominance of bacteria that digest breastmilk and may confer resilience to external insult (86–95). Diversity metrics are a function of overall number of OTU in a sample, and of the abundance of each. Therefore, increased diversity and richness resulting from asymptomatic infections may reflect a disruption of healthy predominance of particular members of the infant gut, or simply the addition of *Campylobacter* OTU to the gut environment, increasing its overall species composition. In contrast, colonization of the environment with *Campylobacter,* resulting in a reduction in the number or abundance of other members, would result in decreased diversity. Caveats in the interpretation of whether gut diversity denotes stability and resilience have led to calls for studies to broaden microbiome studies to evaluate multiple microbial outcomes (96).

In this study, we conducted additional analyses to detect possible species-level associations between infection and microbiota, which revealed several significant indicators of infection stratum. We identified thirteen OTU from four phyla that were indicative of being in the highest stratum of asymptomatic *Campylobacter* infection throughout the first two years of life, several of which showed suggestive or significant associations with growth. Multiple OTU identified here have previously been implicated in pathways of inflammation and weight gain or loss, and while evidence suggests they are important for the modulation of immunity and metabolism, there are health consequences associated with both increases and decreases in their levels. For example, *F. prauznitzii* has anti-inflammatory properties and is decreased among patients with inflammatory bowel diseases (IBD) (97–99), but elevated levels have been associated with obesity in children (100) and in weight loss during fasting (101). *R. gnavus* is common in both infant and adult populations (102,103) and has been suggested to promote protein synthesis by the host gut epithelium and has been associated with weight gain (54); however, it is implicated in IBD when present at higher levels (104). In the current study, both OTU are prevalent across both infection strata, but were significantly more abundant in samples contributed by children experiencing the highest burden of asymptomatic infections, and there was some suggestive evidence that presence of *F. prauznitzii* was associated with reduced linear growth. The role of *P. distasonis,* also indicative of high infection burden, is similarly ambiguous, with prior evidence suggesting a role in both reduced (105) and increased inflammation (106) and colitis (107). *P. distasonis* and two more members of the phylum *Bacteroidetes* (unspecified *Prevotellaceae*) were indicative of high asymptomatic disease burden and associated with growth deficits. Finally, *S. anaerobia* and *S. dextrinosolvens*, both of which were associated with being stunted, are succinate-utilizing bacteria associated with high-starch, western diets (108–112). The mixed evidence as to benefit and harm associated with presence and abundance of these bacteria demonstrates that while optimal levels are not yet identified, these bacteria can impact both inflammatory and metabolic processes in the human gut. The observation that *Campylobacter* burden is associated with differences in these populations is consistent with the hypothesis that asymptomatic infection load may result in alterations to bacterial groups that are noted here and elsewhere to be associated with inflammation and growth. This would further support the EE hypothesis; however, data presented here are insufficient to confirm any precise causal mechanisms. Remaining OTU that emerged as having a potential relationship to growth were largely absent in the literature.

We identified nine OTU indicative of being in the low-burden stratum of asymptomatic *Campylobacter* infection, the majority of which were butyrate-producing

Firmicutes. Butyrate is a short-chain fatty acid produced by the fermentation of fiber in the colon, and a documented energy source for colonocytes associated with improved gut barrier integrity and with well documented anti-inflammatory activity (113–115). Some of these species, such as *A. hadrus* and *E. ramosum,* have nonetheless been associated with undesirable health outcomes such as obesity (116) and diarrhea-predominant IBS (117). We also noted that while *B. breve and B. fragilis,* both adept at metabolizing breastmilk (118), were significantly more abundant in low-burden group at 6 and 12 months, respectively. Exclusive breastfeeding was significantly protective against *Campylobacter* infection in the MAL-ED study (36), and it is possible that this finding is reflective of the greater protection enjoyed by infants whose diet was still largely composed of breastmilk in early life..

Results should be viewed in light of several methodological limitations. First, we were unable to conduct species-level analyses of *Campylobacter* infections. However, given the considerable underestimation of infection by culture detection, which this parent study brought to light (40), we opted to accept this caveat and rely on ELISA methodology to capture the greatest proportion of infection in the population. In light of recent evidence that a broader range of species can cause morbidity than previously known (42), future work should consider whether different species of *Campylobacter* may have distinct consequences for the gut environment and subsequent inflammatory or metabolic sequelae. Our reliance on 16S rRNA data to differentiate gut microbe species precluded us from measuring genomic markers or drawing any inference regarding the function of microbial populations affected by *Campylobacter* infection. Whole genome sequencing should be considered in future studies describe to functional and metabolic profiles of the microbiota and gain a more complete understanding of the functions that may be affected by infection burden (119). Finally, ISA is one of many methods assessing OTU-level changes in the gut. This work did not interrogate patterns of beta-diversity nor employ alternate OTU-level methodologies to assess changes to broader gut bacterial populations.

Nonetheless, we illustrate changes to gut microbial populations associated with asymptomatic *Campylobacter* infections in a longitudinal study with a large sample size and generate testable hypotheses to improve control of this highly prevalent enteropathogen and mitigate its adverse effects on growth in infancy and early childhood. The demonstration that alterations in gut microbial populations associated with *Campylobacter* is independent from diarrhea-related purging and accelerated transit of upper gut flora is notable, as are the findings that different enteropathogens appear to have consistent and differing effects on diversity and membership of the microbial community. These data can inform translational research in order to guide novel interventions.

Table 1: Cumulative *Campylobacter spp* history among 271 children in Peru demonstrates nearly universal asymptomatic infection and considerable burden of diarrheal disease from 0-24m of age

Campylobacter infection & diarrhea	6m	12m	18m	24m
Proportion of children ever infected	46.5 (126)	76.0 (206)	77.1 (209)	92.6 (251)
Proportion of children ever experiencing *Campylobacter* diarrhea	26.2 (71)	57.6 (156)	69.7 (189)	81.6 (221)
Mean (range) of *Campylobacter*-positive diarrheal episodes by ELISA	0.26 (0-4)	0.97 (0-4)	1.9 (0-11)	2.6 (0-15)
Mean (SD) proportion of surveillance stools per child with *Campylobacter* detected	12.6 (17.4)	19.2 (16.9)	21.3 (15.7)	22.5 (15.6)
Mean (SD) proportion of diarrheal samples collected from each child with *Campylobacter* detected	20.5 (31.4)	30.3 (21.9)	32.6 (25.5)	33.2 (24.4)

Table 2: Symptomatic and asymptomatic *Campylobacter* infections and community-level microbial indices

	Mean microbial metric β (SE); *95% confidence interval*			
	Shannon	CHAO1	Phylogenetic Diversity	Simpson's
Asymptomatic infection (per 10% increase in proportion of stools infected)[1]	0.61 (0.18) *0.25, 0.96*	26.61 (10.00) *7.02, 46.21*	2.99 (0.71) *1.59, 4.39*	0.13 (0.03) *0.06, 0.19*
Diarrhea (n of episodes)[2]	-0.01 (0.02) *-0.05, 0.03*	-0.53 (1.16) *-2.80, 1.73*	0.05 (0.08) *-0.11, 0.21*	-0.003 (0.004) *-0.01, 0.01*
Severity (CODA score)[3]	-0.02 (0.03) *-0.07, 0.03*	-0.61 (1.45) *-3.45, 2.24*	-0.06 (0.10) *-0.26, 0.14*	-0.001 (0.004) *-0.01, 0.01*
Duration (days)[4]	-0.03 (0.02) *-0.07, 0.01*	-1.79 (1.19) *-4.12, 0.55*	-0.11 (0.09) *-0.27, 0.06*	0.006 (0.003) *-0.01, 0.00*

Models are adjusted for age, breastfeeding frequency, category, pathogen pressure excluding Campylobacter and antibiotic exposure. Additional adjustment for [1]proportion of surveillance stools infected with other enteropathogens, [2]number of Campylobacter-negative diarrheal episodes experienced, [3] severity and [4]duration of Campylobacter-negative diarrhea was conducted in each model, respectively, to assess whether Campylobacter infection and diarrhea were independently associated with microbial indices.

Table 3: Operational taxonomic units significantly associated with asymptomatic *Campylobacter* burden in the Peru Microbiota Collaboration

Age bin	Indicator Value		Specificity		Fidelity		OTU	Adj. p-value
	I.Low	**I.High**	**A.Low**	**A.High**	**B.Low**	**B.High**		
6	0.00	0.20	0.01	0.99	0.01	0.20	Campylobacter jejuni	0.009
6	0.04	0.35	0.21	0.79	0.21	0.44	Ruminococcus gnavus	0.031
6	0.01	0.21	0.07	0.93	0.08	0.22	Parabacteroides distasonis	0.031
6	0.61	0.34	0.64	0.36	0.96	0.94	Streptococcus infantis	0.031
6	0.54	0.46	0.54	0.46	1.00	1.00	Bifidobacterium breve	0.040
12	0.39	0.02	0.85	0.15	0.46	0.12	Bacteroides fragilis	0.017
18	0.06	0.48	0.21	0.79	0.31	0.60	Faecalibacterium prausnitzii	0.029
18	0.00	0.24	0.04	0.96	0.03	0.25	Lysobacter b01	0.029
24	0.07	0.57	0.25	0.75	0.27	0.76	Uncultured catenibacterium	0.006
24	0.00	0.29	0.10	0.90	0.04	0.32	Prevotellaceae	0.006
24	0.00	0.35	0.02	0.98	0.02	0.36	Prevotellaceae	0.006
24	0.01	0.43	0.10	0.90	0.14	0.48	Holdemanella biformis	0.013
24	0.00	0.37	0.02	0.98	0.12	0.38	Succinivibrio dextrinosolvens	0.020
24	0.15	0.56	0.30	0.70	0.49	0.80	Senegalimassilia anaerobia	0.029
24	0.00	0.26	0.06	0.94	0.06	0.28	Coriobacteriaceae	0.038
24	0.02	0.34	0.15	0.85	0.14	0.40	Phascolarctobacterium succinatutens	0.046
24	0.08	0.53	0.22	0.78	0.35	0.68	Unknown	0.050
24	0.52	0.02	0.92	0.08	0.57	0.24	Erysipelatoclostridium ramosum	0.006
24	0.42	0.02	0.88	0.12	0.47	0.14	Clostridium	0.006
24	0.39	0.00	0.96	0.04	0.41	0.04	Ruminococcus torques	0.006
24	0.57	0.14	0.74	0.26	0.76	0.54	Lachnospiraceae	0.010
24	0.37	0.02	0.89	0.11	0.41	0.16	Lachnospiraceae	0.029
24	0.69	0.29	0.70	0.30	0.98	0.98	Anaerostipes hadrus	0.038

I *variables columns denote indicator values for children in the lowest and highest strata of infection;* ***A*** *(Specificity) is the mean relative abundance of a particular OTU in the lowest and highest strata, normalized to the sum of the means in both strata;* ***B*** *(Fidelity) denotes the total proportion of stools from children in low and high burden groups in which each OTU is present. Species indicative of high and low burden are shown in red and green respectively*

Table 4: Associations between specific OTU and length-for-age Z-score in the Peru Microbiota Collaboration

OTU identified in ISA	Presence β; p-value		Abundance β; p-value	
	LAZ	LAZ + 1m	LAZ	LAZ + 1m
Ruminococcus_gnavus	<0.01; 0.969	0.04; 0.220	<0.01;0.760	<0.01; 0.711
Parabacteroides_distasonis	-0.04; 0.346	-0.03; 0.429	<0.01; 0.919	<0.01; 0.651
Streptococcus_infantis	**0.11; 0.052**	0.06; 0.202	<0.01; 0.925	-0.01; 0.829
Bifidobacterium_breve	NA	NA	**<0.01; 0.045**	**0.01; 0.001**
Bacteroides_fragilis	0.03; 0.589	<0.01; 0.942	1.83; 0.247	0.44; 0.750
Faecalibacterium_prausnitzii	**-0.09; 0.074**	-0.06; 0.136	-1.13; 0.174	-0.60; 0.411
Lysobacter	-0.01; 0.871	-0.01; 0.908	<0.01; 0.954	0.02; 0.691
Catenibacterium	0.01; 0.865	0.06; 0.219	0.02; 0.567	0.01; 0.625
Prevotellaceae	-0.14; 0.100	-0.05; 0.485	-0.09; 0.165	-0.03; 0.623
Prevotellaceae	**-0.14; 0.063**	-0.10; 0.114	-0.13; 0.371	0.01; 0.916
Holdemanella_biformis	-0.05; 0.449	-0.07; 0.248	-0.02; 0.604	-0.03; 0.478
Succinivibrio_dextrinosolvens	**-0.12; 0.068**	-0.05; 0.363	-0.01; 0.284	<0.01; 0.683
Senegalimassilia_anaerobia	-0.02; 0.664	-0.05; 0.237	0.145; 0.222	0.06; 0.594
Coriobacteriaceae	-0.09; 0.234	-0.06; 0.360	0.31; 0.711	0.34; 0.647
Phascolarctobacterium_succinatutens	-0.09; 0.194	-0.04; 0.508	-0.14; 0.422	0.01; 0.949
Unknown	0.02; 0.727	0.02; 0.794	0.08; 0.141	0.01; 0.784
Erysipelatoclostridium_ramosum	**0.10; 0.011**	**0.07; 0.065**	**0.10; 0.009**	**0.09; 0.007**
Clostridium	<0.01; 0.960	0.03; 0.558	0.08; 0.937	1.32; 0.136
Ruminococcus_torques	**0.12; 0.039**	0.08; 0.119	0.04; 0.700	0.01; 0.874
Lachnospiraceae	-0.02; 0.715	-0.05; 0.248	-0.21; 0.799	-0.83; 0.254
Lachnospiraceae	-0.03; 0.625	-0.02; 0.713	0.57; 0.634	1.29; 0.222
Anaerostipes_hadrus	-0.04; 0.443	-0.07; 0.115	0.01; 0.402	<0.01; 0.710

Results of multiple regression modeling association between presence and abundance of OTU on current and subsequent LAZ. OTU are color-coded to represent association with being in the high burden (red) and low burden (green) groups and listed in the same order as Table 3. Models are adjusted for age, LAZ at birth, breastfeeding, antibiotic exposure, dietary diversity, pathogen pressure and diarrheal disease.

Table 5: Associations between specific OTU and odds of being stunted in the Peru Microbiota Collaboration

OTU identified in ISA	**Presence** Odds ratio; p-value		**Abundance** Odds ratio; p-value	
	Stunting at current visit	Stunting at next visit (+1m)	Stunting at current visit	Stunting at next visit (+1m)
Ruminococcus_gnavus	1.00; 0.980	0.88; 0.384	1.02; 0.386	1.02; 0.303
Parabacteroides_distasonis	1.23; 0.148	**1.40; 0.025**	0.92; 0.460	0.97; 0.763
Streptococcus_infantis	0.77; 0.171	**0.72; 0.084**	1.00; 0.961	1.07; 0.540
Bifidobacterium_breve	NA	NA	1.00 ;0.143	**0.99; 0.002**
Bacteroides_fragilis	1.18; 0.352	1.22; 0.255	>100; 0.384	>100; 0.134
Faecalibacterium_prausnitzii	1.12; 0.493	**1.43; 0.03**	4.98 0.581	32.34; 0.233
Lysobacter	0.88; 0.582	0.90; 0.659	0.65; 0.242	0.64; 0.238
Catenibacterium	1.03; 0.892	1.02; 0.904	1.09; 0.361	1.11; 0.239
Prevotellaceae	1.18; 0.551	1.16; 0.605	1.38; 0.315	1.10; 0.679
Prevotellaceae	**1.62; 0.053**	1.43; 0.155	2.03; 0.147	1.31; 0.591
Holdemanella_biformis	1.32; 0.32	**1.57; 0.061**	1.10; 0.547	1.22; 0.232
Succinivibrio_dextrinosolvens	**1.48; 0.068**	**1.50; 0.059**	1.01; 0.880	1.01; 0.827
Senegalimassilia_anaerobia	**1.37; 0.056**	**1.58; 0.005**	0.88; 0.766	0.81; 0.614
Coriobacteriaceae	1.12; 0.663	1.25; 0.385	0.48; 0.821	1.36; 0.922
Phascolarctobacterium_succinatutens	1.20; 0.413	1.05; 0.844	2.03; 0.209	1.40; 0.547
Unknown	0.98; 0.938	0.86; 0.478	0.77; 0.236	0.76; 0.213
Erysipelatoclostridium_ramosum	0.81; 0.143	0.79; 0.104	0.66; 0.189	0.65; 0.183
Clostridium	0.90; 0.583	0.87; 0.463	54.27; 0.231	0.78; 0.939
Ruminococcus_torques	**0.63; 0.030**	0.81; 0.297	0.77; 0.407	0.77; 0.425
Lachnospiraceae	**1.34; 0.086**	**1.54; 0.012**	6.42; 0.515	54.96; 0.151
Lachnospiraceae	0.98; 0.917	1.14; 0.504	2.99; 0.776	0.04; 0.408
Anaerostipes_hadrus	1.20; 0.346	1.33; 0.123	1.03; 0.500	1.01; 0.841

Results of multiple regression modeling association between presence and abundance of OTU on current and subsequent stunting. OTU are color-coded to represent association with being in the high burden (red) and low burden (green) groups and listed in the same order as Table 3. Models are adjusted for age, LAZ at birth, breastfeeding, antibiotic exposure, dietary diversity, pathogen pressure and diarrheal disease.

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Chapter 7: Discussion

Summary of key findings

This dissertation sought to contribute evidence of the hypothesis that disruptions to the gut microbiota of infants living in poverty are implicated in the observed cycle of enteric infections, diarrhea and undernutrition they experience. We employed longitudinal methods to generate population-averaged estimates of associations between metrics of gut microbial maturity, diversity, richness, and presence and abundance of particular taxa with anthropometric indices, diarrheal disease, and *Campylobacter* infections in the largest longitudinal birth cohort interrogating these relationships to date.

This work adds to the literature describing the impacts of age, breastfeeding habits, and dietary diversity on the bacterial composition of the developing infant gut. Our data supports the previously reported finding that the gut microbial community is suppressed by breastmilk in early life, even in the presence of complementary foods, and then undergoes rapid expansion upon weaning. We employ metrics collected through active surveillance that produce near-daily recall and generate detailed dietary and illness histories. In addition to analyzing trends according to traditional breastfeeding categories, we measured frequency of exposure to breastmilk, and found a significant suppressive effect on gut microbial diversity and richness independent of whether children were exclusively breastfed. The use of novel metrics to support prior observations, the frequency of longitudinal measurements, and the large sample size of this cohort allowed us to conduct descriptive analyses that represent an important contribution to the literature, independent of any hypothesis-testing.

We observed suggestive evidence supporting the prior observations that gut microbial maturity, determined by the presence of age-discriminatory taxa in children with healthy growth acquisition, was associated with subsequent weight gain in our population. However, we noted that this cohort was born with and continued to exhibit considerably higher weight-for-length than other populations in which this relationship was observed. Whether this reflects the growing epidemic of overweight and obesity across Latin America (1), or the short length and height characteristic of populations with compounded intergenerational stunting, cannot be deduced from this data. However, we did observe significant evidence of interaction between stunting and wasting, with higher weight-for-length associated with lower length-for-age, which provides support for the latter explanation. In the small subset of children who experienced wasting in this population, we detected pronounced linear growth faltering and a distinct relationship between length-for-age and gut microbial maturity. These data suggest that the gut microbial environment may be reflective of co-occurring and interacting mechanisms driving different nutritional phenotypes.

Our efforts to identify gut microbial markers of linear growth failure did not reveal distinct patterns of microbial maturity, diversity or richness among children with postnatal growth faltering. However, we did identify significant differences in these metrics among children who were born stunted, a condition likely reflective of maternal nutrition both prior to and during gestation (2,3). Children born with intrauterine growth restriction have been identified as a particularly vulnerable subpopulation with significantly increased risk of neonatal and infant mortality (4), as well as continued stunting (5) and its long-term consequences (6). Indeed, the prenatal growth environment is estimated to underlie one fifth of all childhood stunting worldwide (5). We report that LAZ at birth was significantly positively predictive of microbial maturity through two years of age, demonstrating that acquisition of age-appropriate taxa that may be involved in weight gain is compromised in this subgroup. This subgroup of children also experienced significantly compromised trajectories of overall diversity and richness acquisition, with pronounced deficits observed among those children weaned before two years of age.

Together, these observations have implications for transferability of findings between populations. Studies assessing relationships between microbial metrics and child growth, particularly linear growth, have enrolled participants with co-occurring ponderal deficits and have not stratified by, or adjusted for, anthropometric indices at birth. Our data suggest that extrapolating results between populations with different nutritional histories and phenotypes may be invalid, and underscores the need for studies with larger sample sizes that permit stratification of sub-groups and testing of interactions to help elucidate the interplay between length and weight acquisition and the intestinal environment more clearly. Our findings also have programmatic implications for the global health community. While it is not yet clear whether and how microbial populations may be manipulated in effort to ameliorate growth, our findings suggest that more emphasis be placed on the existing WHO recommendation of *continuing* breastfeeding to two years of age to mitigate the gut microbial consequences of insults, particularly among high-risk groups. This recommendation was omitted from recent global nutrition targets (7); work presented here adds further weight to the body of evidence suggesting that duration and not just exclusivity of breastfeeding should be promoted (8).

Given the growing body of evidence supporting associations between the gut microbial community and child growth, to which this study contributed, the subsequent components of our work aimed to test hypotheses that the gut environment is impacted by enteric infection and disease. In a population experiencing the classical cycle of diarrhea and undernutrition, we observed significant reductions in microbial diversity acquisition with increasing frequency, duration and severity of diarrhea, and detected an association between lower gut diversity and increased incidence of diarrheal episodes thereafter (Chapter 5). We further report that apparent consequences of diarrheal disease for the gut environment can endure beyond one month, and that diversity and richness regenerate with increasing time since last insult. While this work was not designed to assess causality, our data illustrate that the microbial community can be impacted in an additive and enduring manner by repeated episodes of diarrheal disease, and that these consequences either cause further vulnerability to disease or are reflective of some underlying mechanism that does. This is the first study to our knowledge that has illustrated this relationship in the context of community-based diarrheal disease in a population with endemic undernutrition. Findings from the first aim of this work (Chapter 4) informed a specific interrogation of whether observed associations differed according to different linear growth profiles. We identified significantly greater impact of diarrheal insults on the gut environment of children born stunted, and observed reductions in the rate at which diversity and richness measures recovered post-diarrhea among children with severe stunting. These data further support the evolving hypothesis that the gut microbial community is involved in the interface between diarrheal disease and child growth.

In an effort to evaluate evidence of the role of gut microbiota as a component of environmental enteropathy, we characterized patterns of diversity, richness, and presence and abundance of bacterial taxa during symptomatic and asymptomatic *Campylobacter* detection (Chapter 6). Infection with *Campylobacter* spp. is thought to be an illustrative example of the EE hypothesis for multiple reasons: it is increasingly understood to drive undernutrition and induce long-term inflammatory and immunological dysfunction, and has been linked to alterations in intestinal permeability and immunotolerance, even in the absence of symptoms (9–17). Prior studies have made important contributions to the understanding of *Campylobacter* epidemiology, demonstrating infection to be associated with significant subsequent linear growth faltering, and employing novel diagnostics to reveal high prevalence of a wider range of *Campylobacter* species than previously appreciated (18–22). Among the 271 children enrolled in the Peru Microbiota Collaboration, we report nearly universal detection of *Campylobacter* in asymptomatic stools, and over 80% prevalence in diarrheal samples from birth to two years of age. Given the well-characterized long-term sequealae of *Campylobacter* spp. across epidemiologic settings, this is a staggering burden of infection and illness. We observed evidence that asymptomatic *Campylobacter* carriage had significant, independent impacts on the gut microbial diversity and richness. Furthermore, we identified 21 specific taxa that were statistically indicative of being in the highest or lowest quartiles of infection burden throughout follow-up, seven of which were associated with length-for-age. Notably, we did not observe as many indicators of other key infections (*Giardia spp.,* entero-aggregative *Escherichia coli* (EAEC), and norovirus) in the population, nor did the taxa overlap with those indicative of these other infections.

Taken together, Chapters 5 and 6 of this dissertation contribute relevant insights to ongoing efforts to disentangle relationships between diarrheal disease, asymptomatic infection, and their intestinal, immunological and systemic consequences. First, we demonstrate that morbidity driven by all-cause diarrhea should not be dismissed as the global health community shifts its focus to EE; while asymptomatic infections, gut permeability and inflammation have clearly emerged as significant components of child health, the physical insult of diarrhea still has important consequences beyond symptomatic illness. Our data show that purging of the gut microbial community that occurs with diarrheal disease, and particularly severe, enduring, or repeated disease, may result in lasting changes to gut diversity and richness. Nonetheless, we also provide evidence supporting supposed pathways of the EE hypothesis. Gut microbiota were identified as correlates of both asymptomatic *Campylobacter* infection and child growth in a population in whom *Campylobacter* is significantly associated with linear deficits. This finding suggests that at a minimum, changes to particular taxa are markers of the posited relationships between *Campylobacter* and stunting in this community; whether microbiota are on the causal pathway between these conditions remains to be determined. That different enteropathogens may elicit distinct changes to the gut community is an important finding with implications for the design of interventions. If pathogens have different impacts on the gut environment, optimal probiotic compounds will be challenging to design, particularly in communities like Santa Clara with a high prevalence of co-infections. Analysis of OTU-level consequences of an array of enteropathogens must be done in order to provide a more comprehensive description of whether and where affected taxa overlap, and importantly whether supplementation designed to encourage expansion of particular groups does not put children at risk of infection, colonization, or pathogenesis with other enteropathogens in circulation.

Methodological and analytic contribution

This study was unique in its methodological and analytic approach, which merits some commentary. We sought to replicate findings that a novel metric of gut microbial maturity was related to wasting among children in LMIC, and assess whether this marker was also sensitive to variations in linear growth. We observed very little variation in the MAZ marker in this cohort, despite the progressive linear growth faltering they experienced. There are various explanations for this negative finding. It is possible that age-discriminatory taxa are not related to linear growth acquisition as strongly as they are to weight gain. It is also possible that the calculation of the metric should be adapted to better discriminate gut flora between children of different growth trajectories. Children selected to create the standard reference group for microbial maturity were included on the basis of never experiencing WLZ or LAZ below -1.75; while this cutoff may be discriminatory for weight in a population with low stunting, it may not be the optimal cutoff to identify taxa that can appropriately discriminate between different linear growth trajectories.

Nonetheless, the use of methods such as random forests to help reduce the multitude of gut microbial data and identify discriminatory taxa will be important to the continuing efforts to identify specific, actionable targets of the gut microbial community that may be implicated in driving undesirable phenotypes. This is particularly true in light of the challenges of relying on judging microbial health as a function of diversity, especially in the context of the rapid changes occurring during infancy. This work, while contributing valuable evidence to characterize patterns of diversity acquisition and its determinants in early childhood, also serves to illustrate these challenges. For example, our observations of significant and enduring deficits in diversity and richness among children with high diarrheal disease burden, children born stunted, or those weaned before the recommended threshold of 24m suggest that deficits confer or reflect negative health outcomes. Indeed, loss of species diversity has been observed in other negative health states in microbiome research. In contrast, the suppressive effects of breastmilk on diversity richness, and the observation that asymptomatic infections increase these metrics, run counter to the narrative that a more diverse gut community is automatically a more resilient one. Furthermore, focusing solely on outcomes to overall metrics of diversity and richness limit opportunities for intervention design; identification of particular taxa that may be conducive to growth or immunity to infection is a necessary step to eventually design clinical interventions. On the other hand, the extraordinary amount of data produced in studies of gut microbial composition and function makes it difficult and infeasible to conduct detailed analyses at the species level across populations with differing dietary and environmental exposures, especially in lower-resource settings. As in all epidemiologic enquiries, there is no single marker or outcome that can be employed to satisfy the many research questions and objectives of this novel field. However, we would argue that in this context, the approach underlying the MAZ score is a very promising one. By identifying taxa that are discriminatory and predictive of various phenotypes, this metric incorporates both community-wide and species-level information. That we did not find this metric to be informative of postnatal linear acquisition in our cohort should provide impetus to reevaluate the parameters used in its calculation and to develop and test it further, rather than to dismiss it.

Finally, we note that this study sought to analytically diversify the landscape of evidence as to the clinical potential of manipulating the microbiota to improve population health. The predominance of cross-sectional studies and panel studies with a focus on a small number of participants has been useful and appropriate for several important reasons. The prohibitive costs of analyzing gut microbial metrics data has, until recently, limited the feasibility of conducting longitudinal studies with large samples sizes. For measures with a high degree of within-person variation, detailed chronicling of trends in a single individual across multiple time points is highly informative. This individual panel design has and will continue to be invaluable to informing translational studies in mice, and studying clinical progression of different gut microbial characteristics. Complementary work describing and comparing microbial features between individuals and communities have been derived from studies enrolling with larger sample sizes, but these have largely been cross-sectional. Together, in vitro and animal models, panel studies, and cross-sectional studies have complemented each other to generate novel hypotheses; however, assessing evidence for these hypotheses in large, longitudinal studies is a necessary next step in the process of epidemiologic enquiry and is crucial to understanding whether we can anticipate population-level health impacts from ensuing research and intervention design. An important overarching aim of this work was therefore to provide a complementary analytical approach to the existing landscape, and inform further iterations of these different types of studies. We did identify population level trends in diversity acquisition, deficit, and rates of regeneration across a large sample of vulnerable children; however, we acknowledge that this work was still largely hypothesis-generating in nature and should inform further translational models to aide in causal inference and in turn provide a justification and basis for the design of therapeutic compounds. Our collaboration with the Gordon Laboratory will permit the extension of our findings to inform experimental animal models. More collaborations with translational as well as epidemiologic components which can continually inform each other in an iterative fashion will be useful to facilitate the eventual goal of delivering tools for the improvement of child health and survival, particularly in vulnerable communities.

Limitations

Findings and contributions must be interpreted in light of several limitations. Gut microbial taxa were identified using PCR primers identifying the V4 region of ribosomal RNA; this methodology has been standard in the literature to date, but its limitations are well documented and recent advances enable more comprehensive and less biased methods. Specifically, the limited precision in identifying bacteria at the species level, as evidence by some of the unknown species emerging in our indicator species analyses, may have precluded us from identifying important taxa. The risk of bias towards amplifying genes from particular groups over others, and the estimation of the phylogenetic diversity of an entire organism based on only one gene segment, may have produced biased estimates of diversity and richness. Whole-genome sequencing should be employed for future studies in order to improve precision as well as expand analyses to characterizing metabolomic and proteomic functions of microbial populations in the human gut, which have been shown to be more preserved across geographic settings than bacterial populations (23). This approach, we expect, will better advance our understanding of whether and how gut microbes may be causally implicated in the relationships observed.

Despite the uniquely comprehensive dataset available to us, there remain variables of interest that we did not measure in this study. First, the absence of microbial measures at birth or enrollment precludes us from understanding temporality of gut microbial and enteropathogen incidence in early life. Second, while the parent study did collect markers of gut inflammation and permeability, the timing of collection was not coordinated with objective of the Peru Microbiota Collaboration, and this prevented us from conducting longitudinal analyses of gut microbial markers and markers of EE. Third, given the relevance of maternal nutrition and microbiota to establishing the infant gut environment, our lack of consideration of these variables prevents us from accounting for features that have been demonstrated to be related both to our exposures and our outcomes. Fourth, given the volatility of the microbial community in this age bracket, more frequent measures of gut microbial indices would aide in understanding temporal relationships and informing causal inference. Ideally, we would have metrics derived for all monthly surveillance stools to align with our detection of asymptomatic carriage and anthropometric measurements. Nonetheless, quarterly sampling on this large of a population represents a considerable advance in the literature. Fifth, this study enrolled 271 children, only 200 of whom were still under follow-up at endline. Loss to follow-up is a common limitation of cohort studies, and in this community it has been noted to occur among children with less stable housing and food availability whose families migrate up and down the river according to the availability of resources. As such, we risk excluding the most vulnerable participants in our catchment area from final analyses, which threatens to bias our results. Finally, the generalizability of this work is likely limited by various factors. Children in this community have distinct dietary and infection histories relative to other settings in LMIC; that these have been chronicled in detail and compared with seven other settings with similar experiences of enteropathy and undernutrition, however, is an attribute conferred by our ability to nest this analysis within a global multi-site consortium (MAL-ED). Generalizability may be more challenging than many other areas of research, given the profound impacts of differential dietary habits on bacterial populations. We therefore reiterate that future analyses should assess functional profiles of gut taxa, rather than species composition alone.

Recommendations for future research

Our study provided evidence of the hypothesized involvement of the gut microbiota in the cycle of enteric infection, illness, and undernutrition among young children at the population level who exhibit high prevalence of stunting and enteropathy. The findings described generate further questions regarding the precise causal mechanisms underlying the observed associations, and the determination of specific and actionable targets to interrupt pathways compounding childhood morbidity and mortality in LMIC. This work should inform the expansion of these analyses to include other sites in the MAL-ED study, a unique opportunity to use shared study design, diagnostic approaches, and methodology to compare large longitudinal cohorts across multiple settings in South Asia, Sub-Saharan Africa and Latin America. Assessing the utility of the microbial maturity marker in these other populations will help to clarify whether our observations were a function of the metric itself or the distribution of weight and length in our cohort.

Similarly, assessing whether compromised diversity acquisition or regeneration among children with intrauterine growth restrictions or severe stunting is replicated in other settings can help verify whether these are generalizable or specific to this population.

Furthermore, translational animal studies can help to clarify whether observed microbial community patterns or specific taxa are causally associated with increased diarrheal incidence or growth failure. For example, given the observation that children born stunted are compromised in their ability to diversify their gut environment, particularly upon weaning, fecal microbiota harvested from their feces may be used to inoculate mice and observe subsequent nutrient harvest and ecological expansion, in the presence and absence of breastmilk. Similarly, taxa identified as indicative of high or low *Campylobacter* infection strata can be used to murine or porcine models to observe their potential impacts on growth acquisition. Finally, we recommend a more detailed analysis of differential effects of dietary components and the timing of their introduction on the gut environment. For the purposes of this work, dietary diversity was largely included as a potential confounder in order to assess evidence of independent relationships between infection, diarrhea, and the microbiota; however, the wealth of data collected in the parent study would allow detailed analysis of how exposure to different food groups and sufficiency of micro and macronutrients can mitigate or exacerbate unfavorable patterns of gut microbial assembly and function in early life. Informing these subsequent analyses is an important output of this project.

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Appendices

Appendix 1: MAL-ED Investigators and Institutional Affiliations

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[3]Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand
[4]Christian Medical College, Vellore, India
[5]Duke University, Durham, NC, USA
[6]Fogarty International Center/National Institutes of Health, Bethesda, MD, USA
[7]Foundation for the NIH, Bethesda, MD, USA
[8]Haydom Lutheran Hospital, Haydom, Tanzania
[9]icddr,b, Dhaka, Bangladesh
[10]Institute of Medicine, Tribhuvan University, Kathmandu, Nepal
[11]Johns Hopkins University, Baltimore, MD, USA
[12]The Pennsylvania State University, University Park, PA, USA
[13]Temple University, Philadelphia, PA, USA
[14]Universidade Federal do Ceara, Fortaleza, Brazil
[15]University of Bergen, Norway
[16]University of Illinois at Chicago, IL, USA
[17]University of Venda, Thohoyandou, South Africa
[18]University of Virginia, Charlottesville, VA, USA
[19]Walter Reed/AFRIMS Research Unit, Kathmandu, Nepal
[20]Haukeland University Hospital, Bergen, Norway

Appendix 2: MAL-ED Forms

2a. MAL-ED Screening & Eligibility Form

SCR/SAR/v1.8/21DEC09

Child ID: ☐☐☐☐☐☐☐☐

MAL-ED

SCREENING FORM (SCR)			
If no response for any question, write NA as response.			
#	Question	Code	Response
01	Study researcher/Nurse/Fieldworker ID		☐☐☐
02	Today's date (DD/MMM/YY)		☐☐/☐☐☐/☐☐
03	Are there plans to move outside of the community within six months?	Yes = 01 No = 00	☐☐
04	Is the mother <16 years of age?	Yes = 01 No = 00	☐☐
05	Does the mother have another child in the MAL-ED study?	Yes = 01 No = 00	☐☐
06	Was this a multiple pregnancy?	Yes = 01 No = 00	☐☐
07	Is the child healthy? (does not have congenital diseases / severe neonatal disease requiring prolonged hospitalization)	Yes = 01 No = 00	☐☐
08	Is the mother able to give informed consent?	Yes = 01 No = 00	☐☐

The child is eligible to participate in the study if:

1) The answer to questions 3, 4, 5, and 6 is No, &
2) The answer to questions 7 and 8 is Yes.

If the child is eligible to participate, continue on to the consent form, or if consent has already been obtained, continue to the Child Assessment Form (CAF).

If the child is **not** eligible to participate (answer to question 3, 4, 5, or 6 is Yes, or answer to question 7 or 8 is No) or if the caregiver is unwilling to answer any of the screening questions, the child **cannot** be enrolled in the study and the Non-Participation Form (NPF) should be filled out with the caregiver.

2b. Baseline Assessment Form

CAF/SAR/v1.11/21DEC09

Child ID: ☐☐☐☐☐☐☐☐

MAL-ED

CHILD ASSESSMENT FORM (CAF)			
If no response for any question, write NA as response.			
#	**Question**	**Code**	**Response**
01	Study researcher/Nurse/Fieldworker ID		☐☐☐
02	Today's date (DD/MMM/YY)		☐☐/☐☐☐/☐☐
03	Screen complete and Consent obtained	Yes = 01 No = 00	☐☐
04	Date of birth (DD/MMM/YY)		☐☐/☐☐☐/☐☐
05	Gender of child	Male=01 Female=02	☐☐
06	Birthweight (kg) * (from birth record, if available)		☐☐.☐☐
07	How long after childbirth did the mother begin breastfeeding him/her?	00 = within 1 hour 01 = 1-24 hours; 02 = 1-3 days 03 = 4+ days later NA = never breastfed	☐☐
08	Was the child fed the first milk (colostrum)?	Yes = 01 No = 00	☐☐
09	Was there pre-lacteal feeding of the child?	Yes = 01 No = 00	☐☐
10	Current weight (kg) *		☐☐.☐☐
11	Current length (cm)		☐☐.☐
12	Current head circumference (cm)		☐☐.☐

* If birth weight OR current weight is less than 1,500 grams, the child is not eligible for the study and the Non-Participation Form should be filled out.

2c. MAL-ED Active Surveillance Form

SAF/SAR/v2.3/31MAY10

Child ID: ☐☐☐☐☐☐☐☐ MMM/YY: ☐☐☐/☐☐

SURVEILLANCE ASSESSMENT FORM (SAF)

01	Date	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
02	Visit today?																															
03	# times																															
04	Field researcher																															
Current health status. Ask about all days since last visit (Yes=1, No=0, NA). [1]Choices for ACTIVITY LEVEL (normal=0, sleepy=1, difficult to awaken=2). [2]Choices for ORAL INTAKE (normal or more=0, less than normal=1). [3]Choices for ANTIBIOTICS (Penicillin=1; Cephalosporins=2; Sulfonamides=3; Macrolides=4; Tetracyclines=5; Fluoroquinolones=6; Unknown=7; Metronidazole=8; Other=9).																																
05	Illness?																															
06	Activity level?[1]																															
07	Oral intake?[2]																															
08	Vomiting?																															
09	Ear pain / pulling?																															
10	Antibiotic use?																															
11	Antibiotic type[3]																															
Gastrointestinal illness. Ask about all days since last visit (Yes=1, No=0, NA). [4]Choices for DEHYDRATED (None=0, Some=1, Severe=2).																																
12	Diarrhea?																															
13	# loose stools?																															
14	Blood in stool?																															
15	Dehydrated?[4]																															
16	ORT administered?																															
17	Sample collected?																															
Respiratory illness. Ask about all days since last visit. If answer to question 18 or 19 is YES in the past 24 hours, look for chest indrawing and record respiratory rate (breaths/minute) twice.																																
18	Cough?																															
19	Short of breath?																															
20	Indrawing?																															
21	Respiratory rate 1																															
22	Respiratory rate 2																															
23	ALRI?																															
Other information. Ask about fever on all days since last visit (Yes=1, No=0, NA). If answer to question 24 is YES in the past 24 hours, record temperature (XX.X °C).																																
24	Fever?																															
25	Temperature °C																															
26	Referral made?																															
27	Nursing notes?																															

Child ID: ☐☐☐☐☐☐☐☐ MMM/YY: ☐☐☐/☐☐

	Date	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
What liquids are the child consuming? (ask about past 24 hours and fill in column corresponding to yesterday's date) (Yes=1, No=0, NA)																																
28	Breast milk																															
29	# times during night																															
30	# times during day																															
31	Infant formula																															
32	# times during night																															
33	# times during day																															
34	Animal milk - Tinned, powdered, or fresh																															
35	# times during night																															
36	# times during day																															
37	Plain water																															
38	Tea, coffee, local examples																															
39	Fruit juices																															
40	Other liquids																															
What solid foods are the child consuming? (ask about past 24 hours and fill in column corresponding to yesterday's date) (Yes=1, No=0, NA)																																
41	Semi-solid, mashed or solid foods? If NO, then stop.																															
42	Porridges, breads, other foods/drinks made from wheat, oat, barley, soy?																															
43	Rice?																															
44	White potatoes, white yams, manioc, other roots?																															
45	Peanuts, spreads with peanuts?																															
46	Beans, peas, lentils?																															
47	Bananas?																															
48	Yogurt, keifer?																															
49	Onions, leeks, shallots, garlic?																															
50	Fermented foods or drinks?																															
51	Supervisor check (initial)																															

2d. MAL-ED Stool Receiving Form

SRF/MM/V12.0/20JAN12

Participant ID: ☐☐☐☐☐☐☐☐

MAL-ED

STOOL RECEIVING FORM (SRF)

If no response for any question, write NA as response.

#	Question	Code	Response
01	Sample ID		☐☐☐☐☐☐☐☐☐
02	Date of collection	DD/MMM/YY	☐☐/☐☐☐/☐☐
03	Time stool specimen was produced	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
04	Time stool specimen was picked up by field worker	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
05	Time stool specimen was preserved in Cary Blair	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
06	Time stool specimen was received at field site/laboratory	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
07	Technician code processing stool specimen	###	☐☐☐
08	Was an unpreserved stool specimen received from field?	Yes=01, No=00	☐☐ *YES = go to question #9.* *NO = complete QNS form and go to question #9.*
09	Type of stool specimen collected	Monthly=M1, Diarrhea=D1, Monthly Recollection=MR, Diarrhea Recollection =DR	☐☐
10	Month of stool collection	##	☐☐
11	If sample is a recollection, what is sample ID of initial stool sample?		☐☐☐☐☐☐☐☐☐
12	Amount of stool received (g)	##.##	☐☐.☐☐
13	Was recollection requested?	Yes, monthly specimen =01, Yes, diarrheal collection =02, No=00	☐☐
14	Consistency of stool specimen	Watery=01, Liquid=02, Soft=03, Formed=04	☐☐
15	Was stool specimen bloody?	Yes=01, No=00	☐☐
16	Did stool specimen have mucus?	Yes=01, No=00	☐☐
17	Was Cary Blair used in field?	Yes=01, No=00	☐☐ *YES = go to question #19.* *NO = go to question #18.*
18	Time Cary Blair used in laboratory	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
19	Was formalin used in the field?	Yes=01, No=00	☐☐ *YES = go to question #21.* *NO = go to question #20.*
20	Time formalin used in laboratory	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
21	Time stool specimen left field site laboratory	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
22	Time stool specimen received at main testing laboratory	Time (24 Hr Scale; HH:MM)	☐☐:☐☐
23	Does stool specimen require a QNS set up?	Yes=01, No=00	☐☐ *YES = complete QNS form.* *NO = STOP, form is complete.*

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SRF Page 1 of 1

2e. MAL-ED Bacteriology Form

BDF/MM/V7.0/16MAY13

Participant ID: ☐☐☐☐☐☐☐☐

MAL-ED

BACTERIOLOGY DATA FORM (BDF)

If no response for any question, write NA as response.

#	Question	Code	Response
01	Sample ID		☐☐☐☐☐☐☐☐☐
02	Date specimen was processed	DD/MMM/YY	☐☐/☐☐☐/☐☐
03	Technician code processing sample	###	☐☐☐
04	Was a culture performed?	Yes=01, No=00	☐☐ *YES = go to question #5.* *NO = go to question #12.*
05	Was Cary Blair used for the culture?	Yes=01, No=00	☐☐
06	Was Aeromonas detected?	Yes, Aeromonas spp=01, Yes, Aeromonas hydrophila=02, No=00	☐☐
07	Was Salmonella detected?	Yes, Salmonella typhi =01, Yes, Salmonella paratyphi A=02, Yes, Salmonella GpB=03, Yes: Salmonella GpC=04, Yes: Salmonella GpD=05, Yes: Salmonella spp=06, Yes: Salmonella cholerasuis=07, Yes: Salmonella gallinarum=08, Yes: Other Salmonella=09, No=00	☐☐
08	Was Plesiomonas shigilloides detected?	Yes=01, No=00	☐☐
09	Was Shigella detected?	Yes: Shigella dysenteriae GpA=01, Yes: Shigella flexneri GpB=02, Yes: Shigella boydii GpC=03, Yes: Shigella sonnei GpD=04, Yes: Shigella sp non-typable=05, No =00	☐☐
10	Was Vibrio detected?	Yes: Vibrio cholerae 01, Inaba=01, Yes: Vibrio cholerae 01, Ogawa=02, Yes: Vibrio cholerae 0139=03, Yes: Vibrio parahaemolyticus =04 Yes: Vibrio (all others)=05, No=00	☐☐
11	Was Yersinia enterocolitica detected?	Yes=01, No=00	☐☐
12	What method was used to test for Campylobacter?	ELISA = 01, Culture = 02 ELISA and Culture = 03	☐☐ *ELISA = go to question #13.* *CULTURE = go to question #14.*
13	Campylobacter ELISA result	Positive=01, Negative=02, Indeterminate=03	☐☐
14	Was Campylobacter isolated?	Yes, Campylobacter jejuni=01, Yes, Campylobacter species=02, No=00	☐☐
15	Technician code performing test	###	☐☐☐
16	Date of test	DD/MMM/YY	☐☐/☐☐☐/☐☐
17	Was E. coli detected?	Yes=01, No=00	☐☐
18	Was E. coli archived for PCR?	Yes=01, No=00	☐☐ *YES = go to E. Coli PCR form.*

Curriculum Vitae

Saba Rouhani

EDUCATION

- **Johns Hopkins Bloomberg School of Public Health** *Baltimore, USA, 2013-2018*

  PhD: Global Disease Epidemiology and Control, Department of International Health

  Thesis: *Gut microbes, enteropathy and child growth: the role of the gut microbiota in the cycle of diarrhea and undernutrition in Peru*
- **London School of Hygiene and Tropical Medicine** *England, UK* 2009-2010

  Master's Degree: Control of Infectious Diseases

  Thesis: *Heterogeneities in malaria infection among Kenyan school-children: implications for case detection strategies*
- **University of Edinburgh***, Scotland, UK* 2005-2009

  Bachelor's Degree in Biological Sciences, Infectious Diseases Honors Program

  Dissertation: *The Implications of Malaria-Helminth Co-infection for Control Strategies in Sub-Saharan Africa*
- **University of Chicago***, Chicago, USA,* 2004-2005

WORK EXPERIENCE

- **Spark Street Consulting** *New York, NY*

  Title: Global health consultant *9/16- ongoing*

  Responsibilities: Health communications management, research and policy brief production, development of indicators, engagement with stakeholders & management of expert reviews

  Key projects: Assembly and participation in the campaign team to support candidacy of Dr. Sania Nishtar for Director General of the WHO in 2017; development of global indicators for violence prevention.
- **Flamingo Global Insight & Brand Consultancy** *London, United Kingdom*

  Title: Global health consultant *4/15- ongoing*

  Responsibilities: Consulting on behavior change strategies for hygiene promotion, uptake of global health interventions
- **Johns Hopkins Bloomberg School of Public Health** *Baltimore, MD*

  i) *Title*: Graduate teaching assistant, *Dept of International Health 9/14-11/14 & 1/15-5/15*

  Responsibilities: Organization of course materials; student mentoring; designing and grading assignments, instruction

  iv) *Title*: Research assistant, *Global Disease Epidemiology and Control, 1/15-ongoing*

Longitudinal analysis, manuscript preparation; data management for cohort study of 300 children; contributing researcher at the MAL-ED consortium (http://mal-ed.fnih.org/) studying enteropathy and undernutrition at eight sites globally

v) *Title*: Research assistant, *Health Behavior & Society, 10/16-ongoing*

Production of grey literature and manuscripts for advocacy on harm reduction strategies to combat US opioid crisis, mass incarceration crisis in Baltimore City.

- **London School of Hygiene and Tropical Medicine** *London, UK*

  Title: Research assistant, *5/12- 5/13*

  Responsibilities: Statistical analyses, literature reviews, manuscript preparation; dissemination for academic and policymaking audiences; grant and proposal writing; logistic planning of upcoming epidemiological studies in Mali, Senegal, and Malawi.

- **Save the Children** *Sikasso, Mali*

  i) *Title*: Research coordinator, *10/10- 5/12*

  Responsibilities: On-site coordination of a randomized controlled trial evaluating the impact of malaria education, insecticide treated bed net distribution and intermittent parasite clearance on biomedical and cognitive indicators in 80 schools in Mali. Tasks included management of sample selection and randomization; community sensitization and consenting; collaboration with health and education authorities at regional and national levels; participation in teacher training and development of educational interventions; development of questionnaires, supervision of data collection and entry; production of final reports in French & English

  ii) *Title*: Consultant in health program evaluation, *9/10- 5/12*

  Responsibilities: Statistical and epidemiological analysis of endline health, nutrition, and behavioral indicators in regions receiving 5-10 years of Save the Children interventions

SKILLS

- Languages: Persian (mother tongue), French (working fluency), Spanish (intermediate)
- Qualitative and quantitative data management and analysis; proficiency in relevant software

AWARDS AND CONFERENCES

- The R. Bradley Sack Family Scholarship Award, Johns Hopkins Bloomberg School of Public Health (2016)
- Save the Children Program Management Award (2012)
- Royal Society of Tropical Medicine and Hygiene Award for Best Poster Presentation of Research in Progress (2012)
- Global Health Established Field Placement Scholarship, Johns Hopkins Bloomberg School of Public Health (2014)

- Posters presented at the Royal Society of Tropical Medicine and Hygiene (2011 & 2012), American Society of Tropical Medicine and Hygiene Annual Conference (2011, 2012, 2016, 2017), Delta Omega Competition (2015)
- Oral presentations at the Comparative & International Education Society Annual Conference (2012 &2013), American Society of Tropical Medicine and Hygiene Annual Conference (2012)

PUBLICATIONS

- Maccario R, **Rouhani S**, Drake T, et al. Cost analysis of a school-based comprehensive malaria program in primary schools in Sikasso region, Mali. *BMC Public Health* 2017; 17(572)
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- Francois R, Yori PP, **Rouhani S** et al. The other *Campylobacter*: Not innocent bystanders in endemic diarrhea and dysentary in children in low-income settings. *PLoS Negl Trop Dis. 2018;12:e0006200.*
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- **Astrovirus often missed in infants with diarrhea worldwide.** Marylynn Larkin**;** Reuters; December 21 2017. *Available at:* https://www.medscape.com/viewarticle/890393

- Lopman BA, Grassly NC. **Editorial Commentary: Pediatric Norovirus in Developing Countries: A Picture Slowly Comes Into Focus.** Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2016. p. 1218–20.