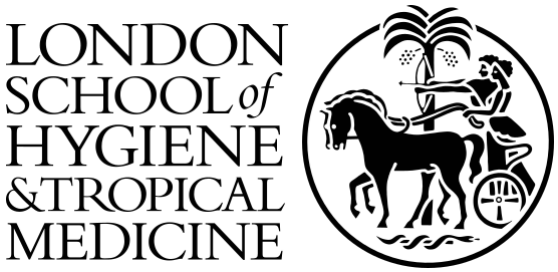

Characterization of the fecal microbiota of rural Malawian children, associations with biomarkers of environmental enteric dysfunction and the impact of a mass drug administration program

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Thesis submitted in accordance with the requirements for the degree of

Doctor of Philosophy

University of London

OCTOBER 2019

The work contained in this thesis was funded by the

Bill and Melinda Gates Foundation

I. Abstract

Environmental Enteric Dysfunction (EED) is a subclinical condition of the gut characterized by changes in gut morphology and function with underlying chronic inflammatory responses. EED affects more than two-thirds of young children living in low-income settings. EED has been linked with growth faltering in young children living in low-income settings, however, the mechanisms by which EED impacts growth are not well defined. A proposed pathway suggests that ingestion of enteric pathogens and toxins, through contaminated food and water, changes gut microbiota composition and function. This results in intestinal inflammation and changes in the intestinal epithelium structure.

There is limited data examining the association between the gut microbiota and EED. The present work was nested in a cluster-randomized trial of mass drug administration of azithromycin and was carried out to characterize composition and diversity of the gut microbiota in rural Malawian children with and without signs of EED. The work also tested for associations between the gut microbiota and stunting. Proteins were extracted from fecal samples and used for the quantification, by ELISA, of neopterin, myeloperoxidase and alpha-1 antitrypsin, which are biomarkers of intestinal inflammation and permeability that have been proposed as potential proxies of EED. Concentrations of the three biomarkers were combined to form a proxy EED score using principal component analysis. Total genomic DNA was also extracted from the fecal samples for V4-16SrRNA sequencing and for species-specific PCR to determine intestinal carriage of bacteria that have previously been shown to be associated with growth in a mouse model.

All three biomarkers decreased with age. *Faecalibacterium prausnitzii* and *Dorea formicigenerans* were prevalent in over 70% of children while *Bifidobacterium longum* was the least prevalent of the bacteria assayed. No associations were found between fecal biomarkers of EED or intestinal bacteria carriage and stunting, however, intestinal carriage of *D. formicigenerans* was associated with normal biomarker concentration. Increased fecal microbiota diversity was associated with a reduction in neopterin concentration. Increased abundance of *Succinivibrio* was associated with reduced EED scores. Mass treatment with azithromycin appeared to have no long-term impact on alpha diversity of fecal samples but was weakly associated with increased abundance of *Prevotella* at 24-months follow-up.

A negative correlation between age and the three biomarkers of EED was found and confirms trends shown in other children living in similar low-income settings. The association between *D. formicigenerans* and normal biomarker concentration suggests a potential beneficial role of the bacterium in gut health. An increase in microbiota diversity is potentially associated with reduced intestinal inflammation, but larger studies are needed to confirm this. The association between *Succinivibrio* and biomarkers of EED shown here is consistent with recent data from another study conducted in Malawi. *Succinivibrio* plays a role in fiber-degradation and metabolites of fiber-degradation have been shown to inhibit intestinal colonization by pathogenic bacteria, therefore, further studies are needed to investigate the significance of this bacterium in EED. Mass treatment with azithromycin may have long-lasting effects on the abundance of defined bacterial taxa but not overall microbiota diversity.

II. Declaration

I, David Chaima, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

David Chaima, October 2019

III. Acknowledgements

It's been a great journey of dedication and hard work, though sometimes challenging. I have gained more invaluable skills and knowledge with the support and contribution from great people. To my supervisors, Prof. Martin Holland and Dr. Sarah Burr: thank you very much for your time, your invaluable support, guidance, patience, encouragement and timely feedback. Your helpful critiques, suggestions and recommendations from the early days to date have helped me complete this work.

I am particularly indebted to Dr. Harry Pickering for his assistance in the lab and throughout the writing process. You were always pleased to assist me whenever I required your assistance. Thank you mate for being selfless. To Prof. Kenneth Maleta: thank you for your mentorship, encouragement and unconditional financial help you provided whenever I was in need. I would also like to thank Dr. Joanna Houghton for helping me a lot in the lab and answering my random questions.

I am grateful to Prof. Robin Bailey and Prof. Khumbo Kalua for the opportunity to do this work within the framework of the MORDOR-Malawi study and for the financial support. I would also like to thank Dr. John Hart, the entire MORDOR-Malawi field team and BICO who collected all the demographic data and biological samples used in this project. In a special way, I would like to thank the participants who agreed to be involved. I would also like to mention Lyson Samikwa and Harry Meleke who supported me in the MORDOR-lab in Malawi and Drs. Rajab Mkakosya and Tonney Nyirenda at the Department of Microbiology, College of Medicine, for their assistance in various aspects.

I would also like to express my gratitude to Eleanor Martins and Esther Amon for the timely arrangements of my visits to London and for all the administrative support. Thanks to Bert, Becca and the whole Trachoma group at LSHTM for assisting me in different ways and making my stay in London fun.

To my lovely wife, Yapoka, thank you very much for your encouragement, emotional support, understanding and constant checks on my progress. This thesis is dedicated to you. Finally, I would like to thank my parents, family and friends for supporting my academic ambitions and for their kind words.

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IV. Index of figures

V. Index of tables

VI. Abbreviations

4plm: 4-parameter logistic model

AAT: Alpha-1 antitrypsin

AGP: Alpha-1 acid glycoprotein

ANOVA: Analysis of variance

CD: Crohn's Disease

CI: Confidence Interval

CRP: C-reactive protein

CSS: Cumulative Sum Scaling

CSV: Comma Separated Values

C_T: Cycle threshold

EAggE: Enteroaggregative *Escherichia coli*

EED: Environmental Enteric Dysfunction

ELISA: Enzyme-linked immunosorbent assay

GH: Growth Hormone

HSA: Health Surveillance Assistant

HAZ: Height-for-age z score

IBD: Inflammatory Bowel Disease

lfcSE: log fold change Standard Error

IGF-1: Insulin Growth Factor-1

IL-6: Interleukin-6

L:M: Lactulose to Mannitol

LAZ: Length-for-age z score

MDA: Mass Drug Administration

MPO: Myeloperoxidase

MUAC: Mean Upper Arm Circumference

NEO: Neopterin

NF-κB: Nuclear factor Kappa B

OD: Optical Density

OR: Odds Ratio

OTU: Operational Taxonomic Unit

PBS: Phosphate Buffered Saline

PC: Principal component

PCA: Principal Component Analysis

PCo: Principal coordinate

PCoA: Principal Coordinate Analysis

PCR: Polymerase Chain Reaction

PERMANOVA: Permutation analysis of variance

PRR: Pattern Recognition Receptor

RUTF: Ready to use therapeutic food

SAM: Severe Acute Malnutrition

SCFA: Short Chain Fatty Acid

SD: Standard Deviation

SRA: Sequence Read Archive

TMB: Tetramethylbenzidine

UC: Ulcerative Colitis

WASH: Water Sanitation and Hygiene

WAZ: Weight-for-age z score

WHO: World Health Organization

VII. Role of the candidate

All demographic and anthropometry data presented in this thesis and the fecal samples used for the various laboratory analyses for this thesis were collected by the MORDOR Malawi study team. The candidate was not involved in the design of the MORDOR Malawi study nor did he play a role in the collection of the data or the fecal samples. The candidate did however clean all the anthropometry and demographic data used in this thesis. The candidate also carried out all of the laboratory work for this PhD thesis. All ELISA assays, DNA extraction from the fecal samples, microbial qPCR and the Bactquant PCR were carried out by the candidate in Blantyre, Malawi, at the College of Medicine, Microbiology Department, in a MORDOR study laboratory. All these assays were new to the MORDOR laboratory; the candidate set up these assays in the MORDOR study laboratory using ELISA and PCR platforms that the candidate helped to set up for other MORDOR study objectives. Library preparation for 16S sequencing was carried out by the candidate in London, UK, at the London School of Hygiene and Tropical Medicine (LSHTM), Clinical Research Department. Sequence data processing in QIIME 2 was carried out at LSHTM by the candidate with guidance from Dr. Harry Pickering. All statistical analyses for this PhD thesis were carried by the candidate with advice from the supervisory team.

1. INTRODUCTION

1.1 Epidemiology of EED

In recent years, nutritionists have paid more attention to environmental enteric dysfunction (EED), a subclinical condition, characterized by impaired nutrition and growth[1]. EED is a disorder of the small intestine characterized by partial villus atrophy, reduced absorptive capacity, increased small intestinal permeability and prominent inflammatory cell infiltration[2,3]. EED, formerly known as tropical enteropathy, was described in the 1960s after an unusual microscopic appearance of the small bowel was reported in adults from low-income countries[4-6] compared to that from apparently healthy adults from Western industrialized countries[7]. Available evidence suggests that EED is more prevalent in low-income countries. A comparison of test results of intestinal permeability and absorptive capacity showed higher levels of intestinal permeability in individuals from low-income countries of South-East Asia and Africa compared to high-income countries of Europe[8]. EED is acquired during infancy and may persist into adulthood however evidence suggests that it is reversible. Expatriates who had EED whilst living abroad in India or Pakistan resolved the signs of this condition after they emigrated to high income settings[9]. Similarly, Indians and Pakistanis with EED indicators who had moved to the USA showed significant improvements in these indicators with increasing periods of residence[10]. EED continues to be prevalent in low and middle-income countries where malnutrition and stunting (impaired linear growth) are more prevalent[11]. In Malawi, approximately two-thirds of children between 3 and 5 years of age have markedly high intestinal permeability typical of EED[12].

1.2 EED pathology

The specific cause of EED is still unclear, however, nutrition, enteric pathogens, enteric toxins, and the gut microbiome[13,14] are some of the factors associated with the etiology of EED. The pathogenesis of EED has been linked with ingestion of fecal-contaminated food and water as a result of poor sanitary conditions. High levels of enteric pathogens found in contaminated food stuffs and toxins produced by the ingested microbes can trigger T-cell hyperstimulation resulting in structural changes in the intestinal epithelium, which include villus atrophy, crypt hyperplasia and increased permeability[15-17]. These changes in the intestinal

epithelium result in malabsorption as shorter villi and deep crypts have fewer absorptive cells[18].

EED does not have immediate symptoms, nor does it have a commonly accepted case definition. Current definitions of EED are based on morphology (short, fused expanded villi compared to long slender villi in healthy subjects as shown in **Figure 1.1.1**), gut function based on biochemical tests such as the dual sugar intestinal permeability test[19], and epithelial architecture (defects in cell adhesion that include loss of tight junctions and intestinal permeability).

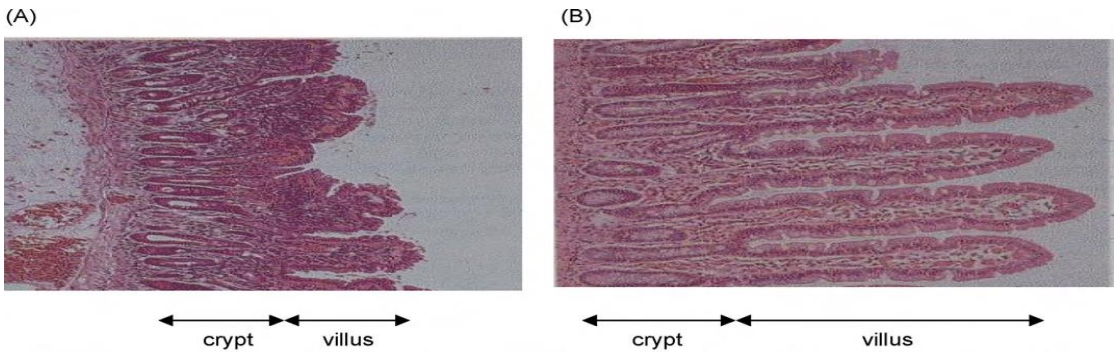

Figure 1.1.1 An illustration of the histological appearance of the small intestinal mucosa of individuals with or without EED. *(A) shows an abnormal EE crypt to villus ratio (B) shows a normal crypt to villus ratio (Figure taken from McKay et al[14]).*

Confirmation of a diagnosis of EED requires evidence of the histological features of EED in the small intestine, which can be gained by endoscopy or biopsy. These are invasive tests that have many logistical challenges and may not be considered ethical in asymptomatic participants. The non-invasive dual sugar absorption challenge test is therefore more commonly used to diagnose EED. The test involves challenging a subject, after a period of fasting, with a solution that has a known concentration of lactulose and mannitol [19,20]. Lactulose is a large sugar that is not normally absorbed by the small intestine while mannitol is a smaller sugar that is absorbed by the small intestine in proportion to absorptive surface area. Both sugars are excreted in urine a few hours after ingestion without being metabolized. As such, urinary mannitol measures absorptive capacity whereas urinary lactulose measures diminished barrier function. Thus, high

urinary lactulose to mannitol (L:M) ratios are an indication of diminished gut barrier function.

1.3 Exposures associated with EED etiology

1.3.1 Nutrition

Intestinal luminal nutrients play a critical role in the maintenance of the morphology and function of intestinal epithelial cells. Both animal and human studies have demonstrated the importance of micronutrients in the proliferation and differentiation of intestinal epithelial cells, which is fundamental to the maintenance of their structure and function. As an example, dietary supplementation of arginine and glutamine was associated with improved intestinal mucosa development of weaned piglets[21]. Similarly, a high protein diet has been shown to upregulate expression of genes involved in cell proliferation and chemical barrier function in rats[22]. Also, zinc deficiency was found to compromise digestive capacity of weaned piglets[23]. In humans, zinc deficiency was associated with increased intestinal permeability,[24] which is one of the characteristics of EED, and treatment with zinc was associated with lower L:M ratios[25]. In brief, these findings suggest that a lack of essential nutrients can lead to impairment of intestinal epithelial function and morphology. Thus, EED may cause nutrient deficiency through inefficient absorption and this may reduce the capability for the intestinal lining to be well maintained, thereby exacerbating EED.

In contrast, there is evidence suggesting that iron supplementation can put one at greater risk of developing EED. A study in Kenya reported that iron supplementation to weaning infants increased the abundance of pathogenic bacteria and also induced intestinal inflammation[26]. In this study, fecal samples were collected from infants at baseline, 3 weeks and 4 months after iron supplementation. Fecal calprotectin, a biomarker of intestinal inflammation, was measured and pyrosequencing was used to characterize the gut microbiota. Pyrosequencing showed a significantly higher abundance of *Clostridium* and *Escherichia/Shigella* and a trend towards lower abundance of genus *Bifidobacterium* in infants with iron supplementation compared to infants without iron supplementation at 4-months follow-up. Additionally, infants who received iron supplementation had significantly higher levels of fecal calprotectin

compared to infants without iron supplementation at 4 months of follow up. Calprotectin is a dimer of calcium binding proteins that accounts for 60% of soluble protein content of the neutrophil cytosol, it is therefore considered a marker of neutrophil influx. Neutrophils are first-line responders to acute-phase inflammation caused by bacterial infection; high fecal levels of calprotectin therefore indicate intestinal inflammation. Taken together, these results suggest a bidirectional relationship between nutrients and EED.

1.3.2 The contaminated environment

There is evidence implicating microorganisms, including parasites, bacteria and viruses, in the etiology of EED. Over a decade ago, the parasite *Giardia duodenalis* was associated with elevated L:M ratios in Nepali infants[27] and Giardia-specific IgM antibody titers were positively associated with L:M ratios in Gambian infants[28]. Similarly, rotavirus infection has been associated with increased L:M ratios in Peruvian children[29] and infection with the bacterium *Citrobacter rodentium* has been associated with reduced villous height seen in jejunal biopsies taken from Zambian adults[30]. Enteroaggregative *Escherichia coli* (EAggEC) infection without diarrhea in Brazilian children has been associated with increased fecal lactoferrin, IL-8, and IL-1b[31]. Fecal lactoferrin, also used as a marker of intestinal inflammation, and IL-1b, a pro-inflammatory cytokine, have been associated with an increase in intestinal epithelium tight junction permeability[32]. Also, *in vitro* studies have associated *Campylobacter jejuni*, a common cause of human bacterial enteritis, with disruption of tight junctions leading to loss of intestinal epithelial barrier function[33].

1.4 The EED pathway and its links to growth faltering

Stunting is very common in Sub-Saharan Africa and elsewhere in low-income countries. It is defined as having a height-for-age Z (HAZ) score more than two standard deviations below the World Health Organization (WHO) child growth standard median[34]. Worldwide patterns indicate that HAZ scores fall dramatically 6 months after birth until 2 years of age in a high proportion of children in low-income countries[35]. It is estimated that nearly 167 million under-5-year old children in low-income countries are stunted[36]. Being stunted has been associated with hindered developmental potential, childhood mortality and morbidity[37,38]. Associations between being stunted in the early years of life and

cognitive performance have also been reported. In Zimbabwean pre-school children, improvements in HAZ scores were associated with increased grade score and early enrollment in school[39]. Likewise, HAZ scores were positively associated with good performance in school[40]. Stunting therefore represents a significant public health concern and as such, its management and prevention have become a WHO global health target in recent years[41].

Stunting is a cumulative process that can start *in utero* and continue during the first 2 years of life. Various factors contribute to stunting *in utero* and during the early years of life. Maternal undernourishment during pregnancy, maternal short stature, inadequate dietary intake, enteric infections and exposure to aflatoxins are some of the known factors that contribute to stunting (**Table 1.4.1**). While it has been suggested that poor nutrient intake contributes to stunting, fortification of complementary foods with micronutrients has largely failed to enhance linear growth or prevent childhood stunting[42-45], which has triggered interest to investigate other causes of growth faltering.

There is a growing belief that EED may be responsible for some measure of growth faltering but the specific mechanism by which this occurs has not been clearly identified. Nutrition, microbes and toxins are some of the suggested key exposures in EED etiology and subsequent growth faltering, however, their role in EED remains unclear. Lunn proposed a causal pathway linking EED to growth faltering[46]. According to Lunn's proposition, increased consumption of microbial pathogens through poor hygiene and sanitation leads to chronic activation of mucosal immunity resulting in intestinal damage and increased permeability. This in turn leads to the translocation of bacteria or bacterial antigens across the intestinal epithelium causing chronic immune activation and the repartitioning of nutrients away from growth towards increased synthesis of antibodies, acute-phase proteins and cytokines. Prendergast and colleagues recently expanded this model by suggesting that the ingested microbes change the composition and function of the gut microbiota resulting in dysbiosis that triggers intestinal inflammation leading to subsequent villus atrophy and impaired enterocyte tight junctions that allow microbial translocation (**Figure 1.4.1**)[47]. The presence of mucosal and systemic inflammation in EED has been supported by recent evidence from a Malawian study characterizing the fecal transcriptome of children

where the L:M ratio test was used to indicate EED[48]. In this study, total RNA was extracted from human cell-enriched fecal samples and thereafter assayed by high-density microarray. The study identified 51 transcripts associated with EED, which were mapped to pathways related to inflammation and cell adhesion. The study also highlighted several immune responses associated with intestinal mucosa damage and upregulation of epithelial tissue repair among others. Intestinal mucosa damage, as a result of chronic inflammation, may expose intestinal microbes to pattern recognition receptors (PRRs) thereby inducing innate immune responses[49] that lead to the secretion of acute phase proteins and pro-inflammatory cytokines, which have been shown to suppress growth[50]. A study in a mouse model in the late 1990s found a negative correlation between insulin growth factor 1 (IGF-1) and the pro-inflammatory cytokine, IL-6[51]. IGF-1 is a hormone that mediates growth hormone (GH)-stimulated somatic growth as well as GH-independent anabolic responses in many cells and tissues. Similarly, a recent study in Zimbabwean infants identified an association between low grade chronic inflammation and suppression of IGF-1 levels where levels of acute phase proteins (CRP and AGP) were higher and IGF-1 levels were lower in stunted infants throughout the first year of life[52]. These findings therefore suggest that elevated levels of proinflammatory cytokines suppress growth. In EED, innate immune responses are not the only important immunological responses; adaptive immune responses are also impacted. T-cell activation, indicated by increased expression of $CD3^+CD69^+$, $CD3^+HLA\text{-}DR^+$ and $CD25^+$ T-cells, is the most apparent adaptive immune response documented in EED and has been associated with crypt hyperplasia, villous atrophy and mucosal damage[3,15].

The above evidence therefore suggests that intestinal inflammation, intestinal damage and systemic inflammation are some of the key features in EED. However, available evidence on the links between these key features of EED and growth faltering is mixed. One multi-site, longitudinal study that assessed the relationship between biomarkers of intestinal inflammation, intestinal permeability and linear growth in infants living in low-income settings of South Asia, Sub-Saharan Africa and Latin America showed that high levels of these biomarkers predicted a decline in infant's length-for-age z (LAZ) scores 6 months after the tests[53]. Similarly, a recent case-control study examining the link between

potential biomarkers of EED and subsequent growth impairment in rural Brazilian children aged between 6-26 months showed an association between higher levels of fecal biomarkers of intestinal inflammation, intestinal permeability and growth faltering[54]. This study reported a significant relationship between high levels of these biomarkers at baseline and poor HAZ scores in the subsequent 2-6 months of follow-up. However, other studies conducted in similar settings have not shown associations between similar fecal biomarkers of intestinal inflammation, intestinal permeability and growth faltering[55,56]. A case-control study conducted in Zimbabwean infants that explored associations between plasma biomarkers of intestinal damage (intestinal fatty acid binding protein [I-FABP]), microbial translocation (soluble CD14 and IgG EndoCAb) and stunting showed no differences in the plasma levels of I-FABP, secretory CD14 and IgG EndoCAb between stunted and non-stunted infants[52]. Similarly, Lin *et al* did not find an association between microbial translocation, indicated by plasma IgG EndoCAb, and HAZ scores[14]. In brief, some of the published data supports the associations between intestinal inflammation and growth faltering[53,54], between systemic inflammation and growth faltering[57,58]and between gut bacterial community structure and intestinal permeability[59] but, the available data is conflicting and therefore not sufficient to make any firm associations between EED and growth faltering. One of the reasons for the discrepancies in the published data could be the differences in the study designs. Longitudinal studies have more often reported significant associations between biomarkers of EED and growth faltering than cross-sectional studies[53,55-57,60].

Table 1.4.1 Factors associated with stunting

Author	Objective	Study design	Findings
Victora *et al.* 2008[37]	To assess the long-term effects of undernutrition on adult human capital, including height, school achievement, economic productivity, and birthweight of the offspring	Meta-analysis review of 5 prospective cohort studies from Brazil, India, The Philippines, Guatemala and South Africa	• Maternal height was positively associated with adult offspring height • Maternal undernutrition during pregnancy was associated with lower birthweight • Maternal intrauterine growth restriction was associated with reduction in birthweight
Umeta *et al.* 2003[61]	To estimate the prevalence of malnutrition and identify factors associated with stunting	Cross-sectional study of breast-fed Ethiopian infants aged 5-11 months	• Infants of mothers with low concentrations of zinc in their breastmilk were more likely to be stunted
Assis *et al.* 2004[62]	To evaluate the relationship between stunting, *Schistosoma mansoni* infection and dietary intake in schoolchildren	Cross-sectional study of Brazilian schoolchildren	• Children heavily infected with *S. mansoni* had a higher risk of stunting than uninfected children • Children with inadequate intake of lipid had an increased risk of stunting compared to those with adequate diets
Gong *et al.* 2004[63]	To assess the effects of aflatoxin exposure on growth	Longitudinal study of 16-37 months West-African children who were residents of villages with low or high aflatoxin exposure. The children were followed up over an 8-month period	• Higher aflatoxin exposure was associated with height retardation over the 8-month follow-up period

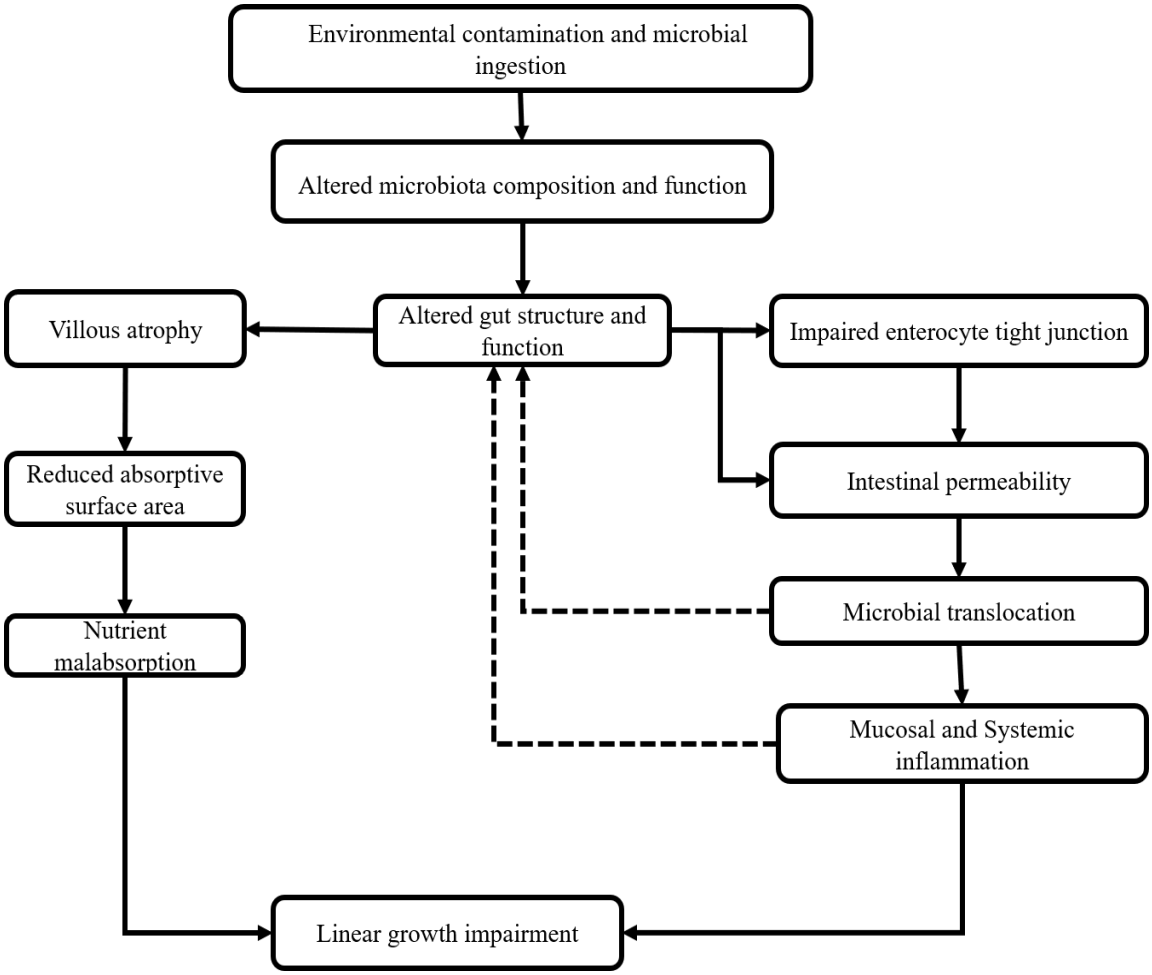

Figure 1.4.1 The proposed EED pathway. *(Figure adapted from Prendergast et al.[34])*

1.5 The gut microbiota

1.5.1 Development of the gut microbiota

Human gut microbial colonization starts at birth and continues to change in species abundance during the breastfeeding period up until the end of complimentary feeding when the microbiota is more adult-like[64]. Gut microbiota composition in infants is influenced by several factors including mode of delivery (vaginal versus caesarian section), breast milk composition, dietary diversity, the environment, and the immune system[65-67]. Available evidence reveals how breastfeeding in infancy influences the composition of an infant's gut microbiota. A study by Bezirtzoglou and colleagues in Greek infants showed breastfed infants to have a more uniform gut microbiota dominated by *Bifidobacteria* and *Lactobacillus* compared to formula fed infants[66]. Similarly, Lee *et al.* reported

higher relative abundance of *Bifidobacterium* in breastfed Korean infants compared to formula fed infants while the phyla *Firmicutes* and *Proteobacteria* were found to be in higher in abundance in fecal samples of formula-fed infants compared to breastfed infants. Another study that compared fecal microbiota composition of exclusively breastfed and formula-fed Dutch infants reported higher abundance of *C. difficile* and *B. fragilis* group in exclusively formula-fed infants. Nonetheless, the infant's gut microbiota is less stable and less diverse compared to that of an adult, which is dominated by *Firmicutes*, *Bacteroidetes* and *Actinobacteria*[64]. A study by Odamaki *et al.* reported an increase in gut microbiota diversity with increasing age that continued sequentially until one's twenties when it stabilized[68].

1.5.2 The gut microbiota and intestinal health

In their reviews, Flint *et al.* and Hooper *et al.* state that the gut microbiota plays a crucial role in nutrient utilization, immune development, and maintenance of the intestinal structure and function[69,70]. Flint *et al.* state that fermentation of nondigestible dietary carbohydrates by the gut microbiota produces short-chain fatty acids (SCFAs), which contribute to the host's energy intake and expenditure thereby maintaining energy balance. SCFAs, which include acetate, butyrate and propionate, also have several biological effects on the host as reported by *in vitro* and *in vivo* studies detailed below. A study conducted in a mouse model to understand molecular mechanisms underlying the modulation of host defense responses and protection against enteric pathogens by commensal bacteria reported an association between an increase in the production of acetate by certain *Bifidobacterium* species and inhibition of an enterohemorrhagic *E. coli* toxin. This effect was mediated by a carbohydrate transporter that was present in certain *Bifidobacterium* strains[71]. In an *in vitro* study using an epithelial cell line, treatment of the cells with butyrate was shown to accelerate the assembly of tight junctions[72]. These findings indicate a potential role of the gut microbiota in preventing colonization by enteric pathogens thereby maintaining gut integrity.

In addition to playing a role in carbohydrate metabolism, gut bacteria also degrade mucins. Mucins are the main structural component of the intestinal protective layer of mucus that also offers attachment sites to intestinal bacteria.

Additionally, mucins are an important source of carbon for the intestinal bacteria at the distal colon, which is made available through mucin degradation by proteolytic enzymes from the host and commensal bacteria. As demonstrated by *in vivo* and *in vitro* studies, the degradation or absence of mucin results in the loss of the barrier function of the intestinal epithelium[73]. There is emerging evidence linking a recently discovered gram-negative mucin-degrading bacterium, *Akkermensia muciniphila*, to gut health. *A. muciniphila* is thought to colonize the intestinal tract early in life with increased abundance within the first year of life, accounting for approximately 1 to 3% of the total microbiota[74]. Since mucin degradation can result in the loss of the barrier function of the intestinal epithelium, the role of *A. muciniphila* in mucin degradation and its abundance in the human intestine warrant further studies.

1.5.3 The gut microbiota and growth

Certain gut microbiota profiles have also been associated with growth phenotypes. A study in Malawi that compared the gut microbiota of mice transplanted with fecal samples from malnourished children to that of mice transplanted with fecal samples from healthy children reported differences in gut microbiota composition between the two groups[75]. A week before transplantation, the mice were fed a sterilized pseudo rural Malawian diet. After fecal transplantation, the mice stayed on the pseudo Malawi diet for 3 weeks and then switched to ready-to-use therapeutic food (RUTF), usually given to undernourished children, for 2 weeks. The mice were switched back to the pseudo Malawian diet after 2 weeks on the RUTF. Mice that harbored fecal samples from malnourished children had higher proportions of *Bilophila wadsworthia* and *Clostridium innocuum* compared to mice that were transplanted with fecal samples from their healthy siblings. Prominent increases in the abundance of *Bifidobacterium* (*B. longum* and *B. bifidum*) and *Ruminococcus* (*R. torques* and *Faecalibacterium prausinitzii*) were also reported in mice that were transplanted with fecal samples from malnourished children compared to recipients of fecal samples from healthy co-twins. The study also reported a reduction in weight within 20 days after fecal transplantation in mice that were transplanted with fecal samples from malnourished twins compared to mice that were transplanted with fecal samples from their healthy co-twins. Another corresponding study reported a causal relationship between gut microbiota

maturity and growth phenotypes in a mouse model[76]. In this study, mice were put on a sterile, pseudo rural Malawian diet, 3 days before fecal transplantation and remained on that diet throughout the study. Mice that were transplanted with fecal samples from healthy Malawian children gained more total body weight and lean mass than mice colonized with microbiota from undernourished children. Additionally, the study also identified specific bacteria associated with weight gain in mice, which included *Bifidobacterium longum, Faecalibacterium prausinitzii* and *Dorea formicigenerans*. The above results from the murine studies indicate an association between a weight loss phenotype and the gut microbiota, which was contingent on the pseudo Malawian diet and the use of germ-free mice. Another study, conducted in Bangladeshi children, that characterized the gut microbiota composition of healthy and malnourished children reported a higher abundance of *Proteobacteria* (which includes pathogenic genera like *Klebsiella* and *Escherichia*) in malnourished children compared to healthy children while *Bacteroidetes* was found to be less abundant in the malnourished children[77]. These findings indicate the potential role of gut microbiota in growth and warrant further studies to investigate the links between carriage of certain bacteria and growth in the human population.

1.5.4 Exposures that affect gut microbiota composition

Living in unsanitary and unhygienic conditions puts children at a risk of ingesting fecal-associated bacteria and microbial toxins. These may be ingested as children play or via contaminated food[78]. Recently, enteropathogen burden has been positively associated with higher L:M ratios[79]. It has been hypothesized that the ingested bacteria may affect intestinal bacterial composition resulting in a pathological shift in the microbiota composition and function in the small intestine[80].

1.5.5 Associations between the gut microbiota and EED

There is limited data suggesting associations between changes in the gut microbiota composition and EED. A recent study that was done in rural Malawian children to identify the gut bacterial community structure associated with intestinal permeability, measured by the L:M ratio test, reported differences in fecal microbiota composition between children with higher and lower L:M ratios[59]. At phylum level, the study found a significant reduction in *Proteobacteria* in children

with higher L:M ratios. At genus level, *Megasphaera*, *Mitsuokella*, and *Sutterella* were found to be more prevalent in children with higher L:M ratios while S*uccinivibrio*, *Klebsiella*, and *Clostridium_XI* were found to be less prevalent in children with higher L:M ratios. These results indicate potential associations between the gut microbiota and intestinal permeability.

1.6 Potential targets for EED interventions

Interventions that may reduce or prevent EED have recently been outlined in a review by McKay *et al.*[81]. In this review, the authors suggest possible interventions that could be used to target the various steps in the EED pathway leading to growth faltering, summarized in **Figure 1.6.1** below.

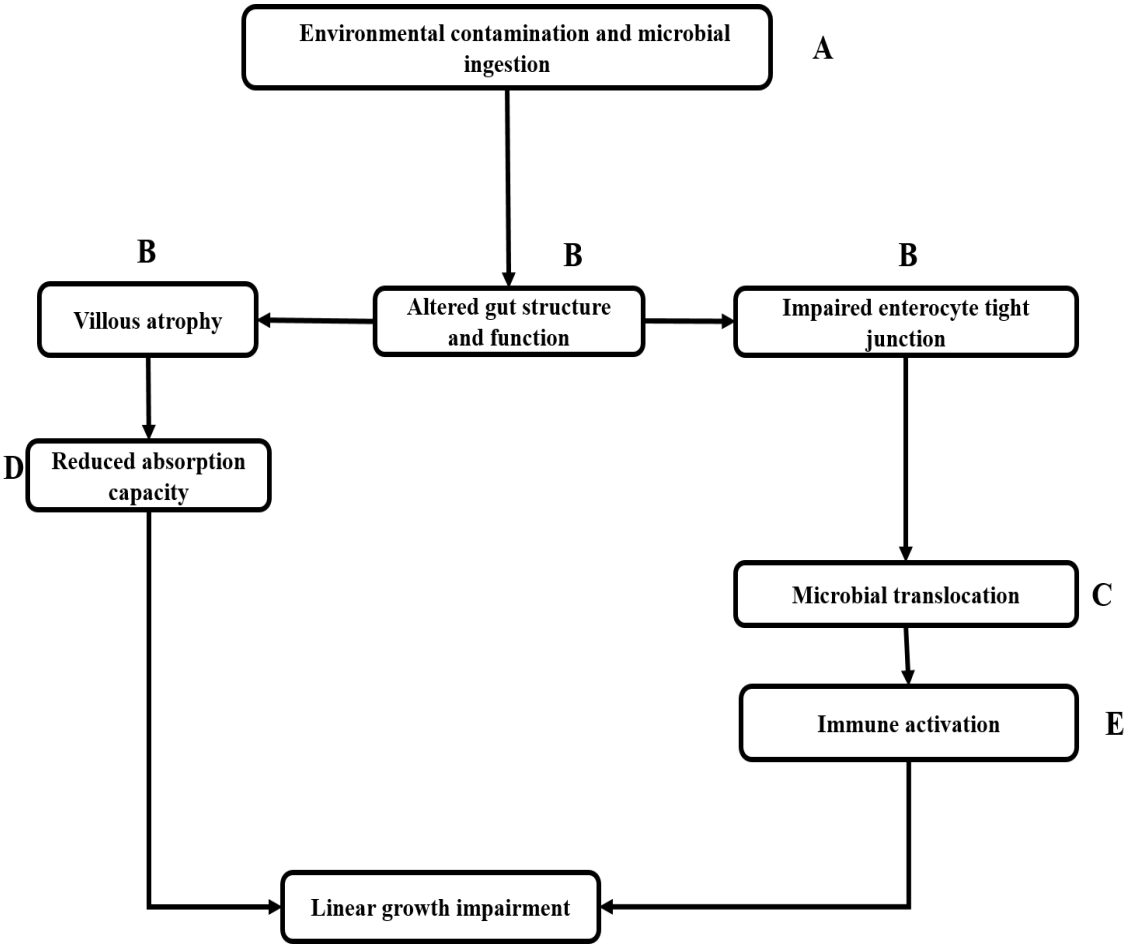

Figure 1.6.1 Schematic showing EED pathway steps that can be targeted by various interventions. *Adapted from McKay et al.[81] (A) Better sanitary conditions and hygiene, (B) Nutrition aimed at maintaining the integrity of the intestinal epithelium, (C) Nutrition aimed at enhancing immune activity, (D) Enhancement of digestion and absorption, (E) Provision of anti-inflammatory drugs/agents to supplement the present anti-inflammatory processes.*

As chronic exposure to fecal pathogens is one of the initial steps in EED etiology, interruption of the fecal-oral pathogen transmission route may be critical in preventing EED. Promotion of breastfeeding in infants, expansion of vaccine coverage by developing new vaccines for enteropathogens, improvement of sanitation and consumption of hygienic food and clean water are some of the suggested ways to interrupt the fecal-oral route[82].

In EED, chronic intestinal responses to enteric pathogens damage the intestinal epithelium thereby impairing gut function. Therefore, nutritional interventions aimed at aiding enterocyte regeneration might help maintain mucosal integrity eventually improving intestinal absorptive capacity. Vitamin A and zinc have potential to improve gut integrity of children living in resource limited settings[83,84] whilst some amino acids have been linked with increased mucin synthesis in infants[85]. As such, these nutrients would be viable intervention options for targeted nutrition to improve gut integrity. Targeted nutritional interventions in EED may also help replenish nutrients that are repartitioned towards immune responses. As an example, intestinal mucosal immune responses involve utilization of amino acids[86]. Therefore, targeted nutritional interventions using proteins would replenish these amino acids as well as help in enterocyte regeneration thereby maintaining gut integrity. Moreover, supplementation of amino acids has been shown to improve intestinal mucosa development in animals[21]. Villus atrophy and crypt hyperplasia that occur in EED are associated with impaired intestinal absorptive capacity as a result of loss of digestive enzymes and presence of fewer absorptive cells. Malabsorption may result in reduced availability of nutrients essential for growth. Provision of pre-digested foods, for instance by fermenting raw materials, and supplementation of foods with digestive enzymes are some of the measures proposed to improve the digestion and absorption of nutrients in EED as some of these methods seem to work in animals[81]. Supplementation of feed with organic acids has been shown to increase villi length in growing pigs thereby improving nutrient absorption[87].

The gut microbiota is one other important aspect of EED etiology. It has been suggested that altered gut microbiota composition may impair metabolism of dietary components[76]. Inefficiency in metabolism of dietary components and an imbalance in mucosal immune responses can affect gut integrity. Since diet has

been shown to shape the gut microbiota[88], nutritional interventions that could promote/support a beneficial gut microbiota profile, such as *Bifidobacterium* and *Faecalibacterium* that have been associated with good growth in mouse models[75,76], may help maintain gut integrity.

Intestinal and systemic inflammation are the most prominent features seen in EED and have been associated with growth faltering[53,58]. One possible explanation for the link between inflammation and growth faltering is that during inflammation more nutrients are directed towards sustaining inflammatory responses leaving inadequate nutrients available for growth and other body processes. The other explanation is that inflammation directly suppresses growth hormones as indicated by studies, described above, that found a negative relationship between pro-inflammatory cytokine[51], acute phase proteins[52] and IGF-1. Hence, use of anti-inflammatory agents/drugs has been suggested as a feasible intervention in EED.

1.7 Tested EED interventions

1.7.1 Water, sanitation and hygiene (WASH)

There is growing interest in how WASH interventions might support strategies to reduce EED and stunting in affected settings. Drawing from evidence reported by animals studies[89,90], Mbuya and Humphrey suggest that improving hygiene and sanitation plus ensuring a clean infant feeding and play environment may interrupt specific pathways through which feco-oral transmission occurs in the first two years of life[91].

There are several interventional studies currently going on in sub-saharan Africa (Kenya, Tanzania, South Africa and Zimbabwe), parts of Asia (Bangladesh, India, Nepal and Pakistan) and South America (Brazil and Peru) aimed at identifying the contributions of WASH interventions to EED and infant growth and also assessing the sustainability of the implemented practices[92-94]. Results from these studies are expected to give a better understanding of the benefits of WASH interventions aimed at reducing both EED and stunting. A study in Kenya that assessed the impact of WASH interventions, directed to mothers, on diarrhoea and linear growth in their children found no association between WASH and linear growth[95]. Similarly, WASH interventions did not prevent enteric infections in rural

Zimbabwean infants[96]. These results highlight the need for more alternative intensive approaches when implementing WASH interventions to successfully interrupt the fecal-oral route.

1.7.2 Targeted nutrition

There have been many studies of nutrition and stunting reported but few of nutrition and EED[81]. However, the few studies done on nutrition and EED have reported mixed results. A study done in malnourished Brazilian children aged 2 to 60 months reported slight improvements in L:M ratios at 10 days follow-up in children who received formula supplemented with glutamine or glycine for 10 days compared to children who received non-supplemented formula[97]. Another study investigating the impact of nutrient supplementation, coupled with an anthelminthic, on EED in rural Malawian children reported no impact[98]. In this study, Malawian children aged 12 to 35 months received a placebo or a single dose of albendazole at enrollment, a daily dose of zinc for 14 days and multiple micronutrient supplement for 24 weeks. L:M ratios were then measured in urine collected at 12-and-24 weeks of follow up. The study reported no differences in L:M ratios at either follow up visit and called for alternative interventions. Currently, clinical trials evaluating the effect of common beans or cowpeas on gut barrier function and growth are being implemented in Malawi[99]. These trials will administer pre-processed legumes to enhance their digestion and absorption and the results are expected to elucidate the potential of these two commonly grown grain legumes on gut health and growth in children at risk of developing EED.

1.7.3 Anti-inflammatory agents

Available evidence supporting the association between gut inflammation and growth faltering[53,54] has triggered research into the use of anti-inflammatory agents as a remedy for EED. A trial of stunted Kenyan children with severe acute malnutrion (SAM) tested the role of an anti-inflammatory agent on EED and child growth. In this trial, children were assigned to treatment with mesalazine or placebo for 28 days and HAZ scores were calculated at baseline, 28 days and 56 days of follow-up. EED was indicated by biomarkers of intestinal inflammation (fecal calprotectin), bacterial translocation (endotoxin) and systemic inflammation (C-reactive protein [CRP]). There were no differences in linear growth between the two groups at 28 days or 56 days of follow-up, however, baseline

concentrations of Insulin-like growth factor-1 (IGF-1) had a strong negative correlation with CRP. This became less apparent in the mesalazine group at later time points suggesting that mesalazine had achieved modest reductions in CRP[60]. Although children were not followed up for an extended period of time in this study, the finding still warrants further investigations of gut specific immunomodulatory therapies targeted at chronic immune activation in order to address EED and stunting.

1.7.4 Antibiotics

Early studies comparing animals given prophylactic antibiotics in their feed versus untreated animals reported growth-promoting effects of antibiotics[100-102]. Recent evidence gathered by meta-analysis and systematic review of randomized clinical trials suggests that prophylactic antibiotics and antibiotics given for infection have growth promoting effects in pre-pubertal children living in low-income countries, although the effect is more pronounced for weight than it is for linear growth[103]. Additionally, the prescription of antibiotics for infection in infants living in low-income settings of South Asia, sub-Saharan Africa and Latin America has been reported to increase weight in the early years of human life[104]. Infants treated with antibiotics in the first 6 months of life had higher weight-for-age z (WAZ) score from 6 to 24 months compared to untreated children in a 24-month follow-up period. The specific mechanism(s) by which antibiotics promote growth is/are not clearly established. One hypothesis is that antibiotics mediate growth by clearing enteric pathogens or by modulation of the intestinal microbiota[105,106]. However, studies looking at the impact of antibiotics on biomarkers of EED have reported mixed results. A study in a rat model reported a reduction in intestinal bacterial load and a change in intestinal microbial profile followed by reduction in mucosal inflammation after 10 days of treatment with rifaximin[107]. In humans, short treatment with rifaximin did not improve L:M ratios in children with EED[108]. Grassly *et al.*, recently reported that short term treatment with azithromycin aimed at improving immunogenicity of oral polio vaccine significantly reduced fecal biomarkers of EED[109]. In this study, infants were randomized to receive a 3-day treatment with azithromycin or placebo and fecal levels of myeloperoxidase, calprotectin, neopterin and alpha-1 antitrypsin were quantified at baseline and 14 days after treatment in both groups. The prevalence of enteric pathogens was also determined at baseline and at 14 days after

treatment in the intervention and placebo groups. The study reported significant decreases in fecal levels of myeloperoxidase, alpha-1 antitrypsin and calprotectin in azithromycin treated infants. Significant reductions in the prevalence of Enteroaggregative, enteropathogenic, and shiga-toxin-producing *Escherichia coli* and *Campylobacter* were found in the azithromycin group compared to the placebo group 14 days after treatment, which correlated with the reduction in the fecal biomarkers. Whether or not these effects persist for a longer time period is not known.

1.8 The potential role of azithromycin treatment on child health

Azithromycin is a broad spectrum, macrolide antibiotic characterized by a long intra- and extra-cellular half-life. Its use is indicated in the treatment of atypical pneumonia, skin and soft tissue infections and sexually transmitted infections[110]. Mass azithromycin treatment at the community level is a corner-stone of the WHO-recommended strategy for control of ocular infection with the intracellular bacterium *Chlamydia trachomatis*, the etiological agent of trachoma[111]. Available evidence from studies of mass azithromycin distribution (MDA) for trachoma control in trachoma endemic areas indicates that mass azithromycin treatment could have secondary effects, which include reductions of child morbidity and mortality[112-114]. In The Gambia, azithromycin MDA for trachoma was shown to have favorable short-term effects on childhood morbidity, including diarrheal disease, in rural areas[115] whilst in Tanzania, reduced risks of diarrhea and acute lower respiratory infections related to azithromycin MDA for trachoma control were reported in young children[113,116]. A study in Ethiopian children between 1 and 9 years of age showed an association between MDA of azithromycin for trachoma control and reduced mortality[114]. In this study, childhood mortality was reduced in communities that received azithromycin compared to control communities. Interest in these results led to a multi-site trial, conducted in Malawi, Tanzania and Niger, which most recently reported lower mortality rates in children under 5 years of age living in low-income settings who received mass azithromycin treatment compared to recipients of a placebo[117]. The study reported a 6% lower mortality rate in Malawian children who received azithromycin compared with placebo, 18% lower mortality rate in Niger children who received azithromycin compared to placebo recipients, and 3% lower mortality rate in Tanzanian children who received azithromycin compared to

placebo recipients. The specific mechanism through which azithromycin reduced mortality in children is not understood, however, available evidence suggests that prolonged mass azithromycin distribution can affect carriage of pathogenic bacteria. For example, azithromycin MDA reduces the abundance of *Campylobacter* spp. in the gut[118] and reduces the community burden of nasopharyngeal carriage of *Streptococcus* pneumoniae[119].

Azithromycin has both antibacterial and immunomodulatory properties and as such azithromycin treatment may provide insight into the etiology of EDD. Inhibition of protein synthesis, disruption of bacterial quorum sensing[120,121] and interruption of adherence to host cells[122,123] are the main antimicrobial mechanisms of azithromycin while modulation of the nuclear factor kappa B (NF-κB) pathway is the main immunomodulatory mechanism[124,125]. The NF-κB pathway regulates pro-inflammatory responses among other functions. A recent study that investigated the effect of one round of mass oral azithromycin treatment on conjunctival immune responses in 6 to 10 year old Tanzanian children reported a downregulation of pro-inflammatory cytokines and chemokines 3 months after treatment underscoring the immunomodulatory effects of azithromycin[126]. There is also evidence on the impact of the antimicrobial effect of azithromycin on the gut microbiota. In the clinical trial of azithromycin by Grassly and colleagues described above[109], the gut microbiota was characterized at baseline and 14 days post-treatment in fecal samples from infants who were randomized to a 3-day course of azithromycin or placebo. The study reported a reduction in microbiota richness, a reduction in relative abundance of phyla *Proteobacteria* and *Verrucomicrobia* and a reduced risk of colonization by *E. coli* and *Campylobacter* in azithromycin treated infants. Another study that investigated the impact of macrolide (azithromycin or clarithromycin) use in early childhood reported that the antimicrobial effect on the microbiota is long lasting[127]. The study characterized the fecal microbiota in fecal samples collected from Finnish children aged between 2-7 years, which were grouped according to antibiotic use as follows: children who had not used antibiotics for at least 2 years but had used antibiotics frequently during early life, children who had used macrolide antibiotics within 6 months of sample donation, children who had used macrolide antibiotics within 6–12 months of sample donation, children who had used macrolide antibiotics within 12–24 months of

sample donation and children who had not used antibiotics for a period of more than 2 years in addition to limited antibiotic use in the early years of life (control samples). Here a reduction in microbial richness was detected in fecal samples from children who had used macrolide antibiotics within 12–24 months of sample donation compared to control samples. The study also reported a significant decrease in the relative abundance of *Actinobacteria* and an increase in *Bacteroidetes* and *Proteobacteria* in fecal samples from children who had used macrolide antibiotics within 6 months of sample donation compared to controls. Nonetheless, these phyla-specific changes were not observed in the groups that had used macrolide antibiotics within 6–12 months and 12-24 months before sample donation. The above results reporting varying persistent effects of azithromycin on the gut microbiota together with the results reported by Grassly *et al.*[109]on the reduction of fecal biomarkers of EED following a short course of azithromycin treatment warrant further research to look at short and long-term effects of azithromycin on the gut microbiota in the context of EED.

2. HYPOTHESES AND RESEARCH QUESTIONS

The hypotheses and research questions below were posed to characterize the fecal microbiota in rural Malawian children, test for associations between the fecal microbiota and biomarkers of EED and assess the long-term impact of mass azithromycin treatment on the fecal microbiota:

2.1 Fecal biomarkers of gut function are associated with growth in Malawian children

2.1.1 What is the magnitude and prevalence of intestinal inflammation and permeability in rural Malawian children?

2.1.2 Are fecal biomarkers of intestinal inflammation and permeability associated with growth outcomes in rural Malawian children?

2.2 Intestinal bacterial carriage of mucolytic bacteria and other bacteria previously associated with growth in a mouse model is associated with biomarkers of EED

2.2.1 What is the prevalence of intestinal carriage of mucolytic bacteria and bacteria associated with weight gain in a mouse model in children living in rural Malawi?

2.2.2 Is there a relationship between fecal carriage of these bacteria and growth outcomes in rural Malawian children?

2.2.3 Is there a link between intestinal carriage of these bacteria and intestinal inflammation and permeability in rural Malawian children?

2.3 Gut microbiota profiles in children with signs of EED are different from children without signs of EED

2.3.1 What are the phylogenetic groupings of bacteria found in children with intestinal inflammation?

2.3.2 Do certain phylogenetic groupings predict intestinal inflammation?

2.3.3 Does a relationship exist between phylogenetic groupings that predict intestinal inflammation and stunting?

2.4 Azithromycin treatment, given in a mass drug administration program, has a persistent impact on gut microbiota composition

2.4.1 What is the effect of azithromycin treatment on the gut microbiota after two and four biannual azithromycin administrations?

3. METHODS

3.1 Details of the demographic and anthropometry data used and biological samples tested

3.1.1 An overview of MORDOR-Malawi

This PhD project utilized data and biological samples collected by investigators during the course of the Mortality Reduction after Oral Azithromycin in Malawi study (MORDOR-Malawi). MORDOR-Malawi consisted of a series of studies investigating the potential for treatment with the broad-spectrum antibiotic azithromycin to reduce childhood mortality and morbidity. The main MORDOR-Malawi study was the MORDOR-Malawi mortality trial, a double-blind, placebo-controlled, cluster-randomized trial designed to test the hypothesis that MDA with azithromycin reduces mortality in children under five years of age. The trial was conducted in Mangochi, Malawi between May 2015 and June 2017 and involved 4 bi-annual rounds of MDA. Three hundred and thirty-four clusters were randomized to receive MDA with either azithromycin or placebo given to children aged 1 to 59 months. A cluster was defined as the catchment of one Health Surveillance Assistant (HSA). Childhood mortality was assessed through six-monthly census rounds that recorded all 1 to 59-month-old children in the study area and any deaths that occurred between rounds. The primary outcome was all-cause mortality.

Running alongside the MORDOR-Malawi mortality trial was MORDOR-Malawi morbidity, a parallel trial designed to explore mechanisms by which azithromycin MDA may reduce childhood mortality and to monitor the emergence of antimicrobial resistance. MORDOR-Malawi morbidity study was also a double-blind, placebo-controlled, cluster-randomized study that collected biological samples in repeat cross-sectional surveys. MORDOR-Malawi morbidity was conducted in a separate set of 30 clusters that were also randomized to receive 4 bi-annual rounds of MDA with either azithromycin or placebo given to children aged 1 to 59 months. Within these clusters, the effect of azithromycin on anemia, malaria parasitemia, carriage of respiratory and gastrointestinal pathogens, trachoma and nutritional status was assessed through biological sampling and anthropometry measures. This PhD project utilized demographic data, anthropometry data and fecal samples collected in these 30 clusters.

3.1.2 MORDOR trial setting

Malawi is a low-income country with a total estimated population of 17.6 million as of 2018[128]. Only 30% of the total population live on more than $1.90 a day[129]. Access to sanitation is poor, particularly in the rural areas; 63% of the total population live in households with improved sanitation facilities (a facility that hygienically separates human excreta from human contact) but only 39% of households in rural areas use improved sanitation facilities[130]. A recent Malawi healthy key indicators survey reported 75% coverage of early childhood vaccinations in Malawian children less than 2 years of age. While breastfeeding is high in early infancy (81% of Malawian infants at 0-1 months of age are reported to be exclusively breastfed), exclusive breast feeding in older infants is low such that only 34% of infants at 4-5 months of age are exclusively breastfed. Over 50% of Malawian children between 6-49 months of age in rural areas are stunted[131].

MORDOR-Malawi took place in Mangochi, a lakeside district located in the South-Eastern region of Malawi. It is one of the most populated, poorest districts of Malawi with high birth rates and low literacy levels[128,131]. A recent study of carriage of enteric viruses and parasites in infants in the district reported over 60% prevalence of Enterovirus, 28% Rhinovirus, 23% Parechovirus, 24% Norovirus, 3% Rotavirus, 10% *Giardia lamblia* and 6% *Cryptosporidium*[132]. Prevalence of intestinal helminths in children in the district is low; there is less than 8% prevalence of *Hookworm* spp., *Taenia* spp., *Ascaris lumbricoides, Strongyloides* and *Trichuris trichura*[133,134].

3.1.3 Selection of clusters and participants in MORDOR-morbidity

For logistical purposes, MORDOR–Malawi split the district into 5 geographical zones namely Monkey Bay, Mangochi, Namwera, Makanjira and Chilipa (**Figure 3.1.3.1**).

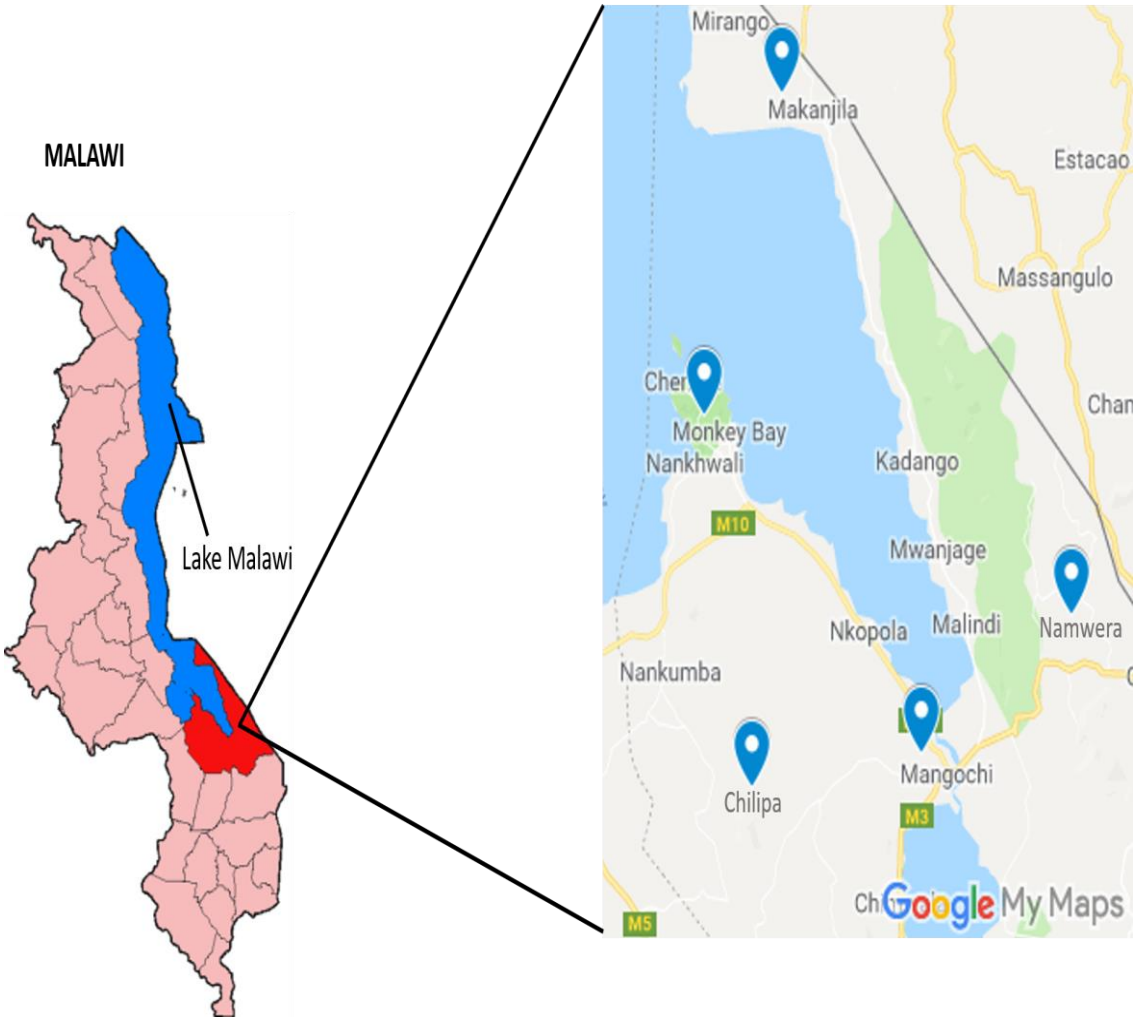

Figure 3.1.3.1 Map of Malawi and the five study zones in Mangochi district. *Mangochi district is marked in red and the five study zones in Mangochi district (Mangochi, Chilipa, Monkey Bay, Makanjira and Namwera) are marked by location icons.*

MORDOR-Malawi morbidity was conducted in 30 clusters that were randomly selected such that each of the 5 geographical zones were represented by 6 clusters. Half the clusters were randomly allocated 4 rounds of bi-annual MDA with azithromycin, and the other half to 4 rounds of MDA with placebo. Eligible communities met the following criteria: (i) the community was in the Mangochi district and identifiable as within the catchment of an HSA, (ii) the community leadership consented to participation (this did not remove the need for individual consent, but without overall leadership consent, the community could not participate), (iii) had an estimated population of between 200–2,000 people and (iv) did not fall within an urban area. The allocation of community assignments was conducted using simple random sampling assigned from the same list of eligible communities.

Before children were randomly selected and recruited for biological sampling, field workers visited the village and collected/updated census information including dates of birth, sex, village location. A random sample of 50 children per cluster was then generated using a computer-generated simple random sample with the aim of recruiting 40 children from each cluster. If the target of 40 children could not be reached from the initial randomization list of 50 children, either due to absence or non-participation, additional children were randomly selected from the census list until 40 children in each cluster were recruited. All recruited children had to meet the following criteria to be eligible to participate in the study: (a) be a resident in the study community, (b) be aged between 1 and 59 months and (c) have a parent or guardian willing to give consent. Children less than 3.8kg in weight were excluded.

The following were collected from recruited children at baseline, 12 and 24-months surveys: fecal samples, dried blood spots, nasopharyngeal swabs, conjunctival swabs, and anthropometry measurements. Enrollment, participation in fecal sample collection and samples included in the analyses of this thesis are shown in **Figure 3.1.3.2** below.

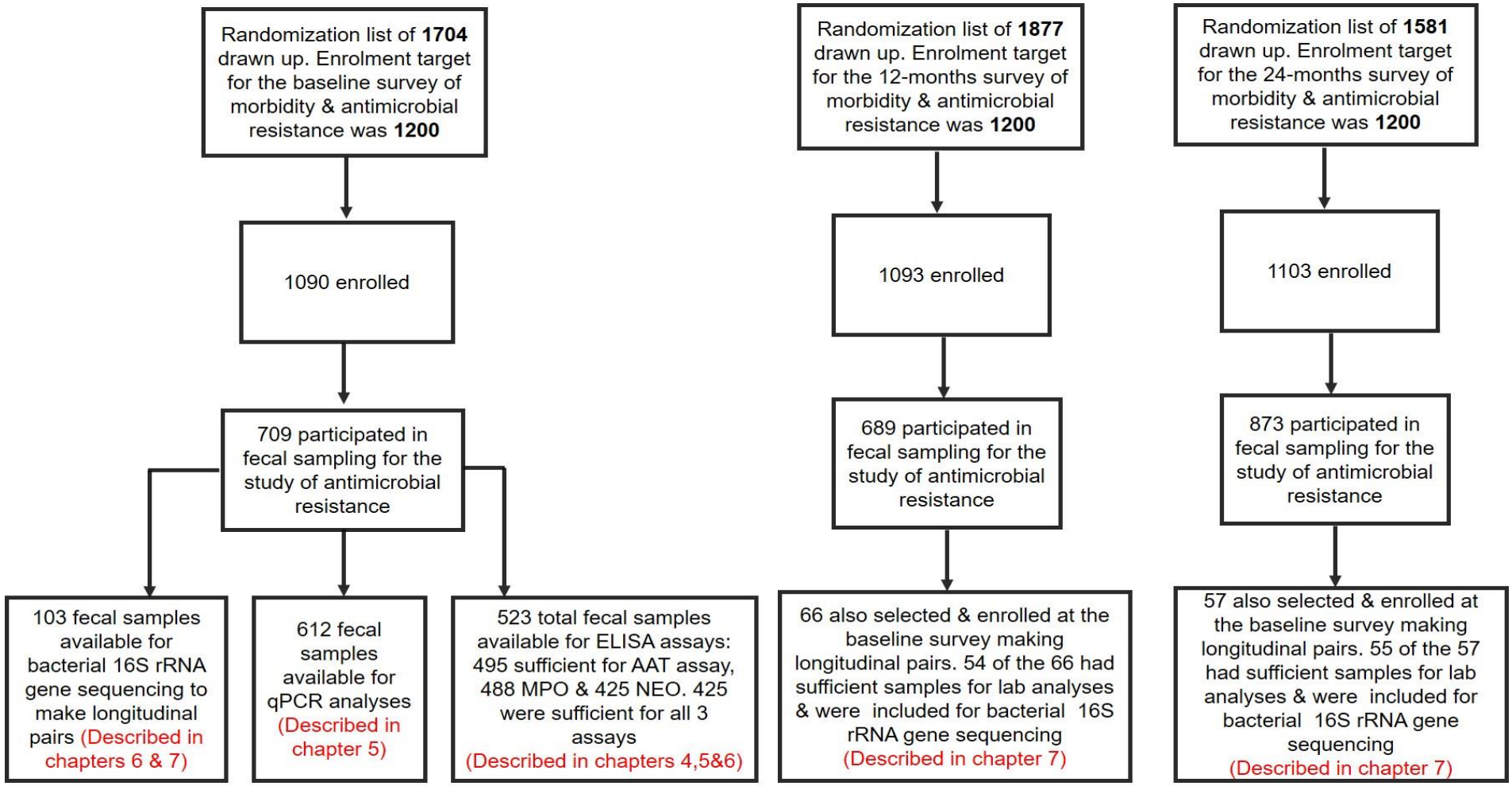

Figure 3.1.3.2 Flow chart of the children enrolled and included in the analyses of this PhD. *The figure shows the number of children selected by MORDOR-Malawi Morbidity, children enrolled, children who provided fecal samples and children included in the final analyses of this PhD.*

3.1.4 Azithromycin / Placebo administration

Children aged 1-59 months on the current census were offered weight- or height-based, directly observed, oral azithromycin suspension (or oral placebo). Children under 1 year of age were weighed and received a dose of 20mg/kg body weight. Children over 1 year of age were dosed according to their height as performed in trachoma control programs[135]. Individuals allergic to macrolides or azalides were not given the study drug but were included in the outcomes. Treatment was given by the HSA of the community.

3.1.5 Fecal sample collection and storage

Fecal samples for participating children were collected by their mothers or guardians. Mothers or guardians were provided with fecal collection kits and given verbal instruction, by a field nurse, on how to collect the sample. Fecal samples were collected into pre-labelled, sterile 50ml falcon tubes without preservative. Mothers or guardians were asked to return the falcon tube with the fecal sample, sealed in a Ziploc bag, to the field team as soon as possible after collection. These samples were put in a cool box with ice packs until the end of the day (not more than 8 hours). If a sample was not collected by the end of field work in that village, field workers returned the following day with a cool box to collect any remaining samples. Samples were brought to the laboratory at the end of the day and stored at -80°C.

3.1.6 Anthropometry measurements

The SECA 877 scale (Chasmors Ltd., London, UK) was used to weigh infants and children to the nearest 0.1 kg. Children were weighed with no shoes, no hair ornaments and no jewelry. If called for, children wore light clothes to protect their modesty. If a child was less than 2 years of age or otherwise unable to stand on the scale unaided, s/he was held by a parent or guardian and the tare function was used to subtract the weight of the adult.

The SECA Leicester height measure (Chasmors Ltd.) was used to measure height for children who were ≥2 years of age and able to stand whereas the SECA 417 infantometer (Chasmors Ltd.) was used to measure recumbent length for

children who were <2 years of age or unable to stand unaided. Both height and length were measured to within 0.1 cm.

To monitor the accuracy of the weighing scales over time, a 5 kg test weight (Chasmors Ltd.) was weighed at the beginning and end of each day. All measurements were taken in triplicate by field nurses.

3.1.7 Ethical considerations

The MORDOR-Malawi study was approved by the London School of Hygiene and Tropical Medicine (UK) and the College of Medicine Research Ethics Committee (Malawi). Use of samples and data collected by MORDOR-Malawi for this PhD project was also approved by both boards (Appendix).

During the MORDOR-Malawi study, consent was first obtained at the community level through discussions with the village chief and community elders who then indicated the willingness, or unwillingness, of the community to participate through verbal consent. Information and consent forms for individual recruitment were translated into local languages (Yao and Chichewa) prior to their approval by the local ethics committee. These were read aloud, by project staff fluent in the local languages, to parents or guardians of all children during the sensitization process, prior to recruitment. Written, informed consent (by thumbprint or signature) was obtained from the parent or guardian of each child before recruitment. During the consenting process, all parents and guardians were informed of their freedom to withdraw their child from the study at any time without giving any reason for doing so.

3.1.8 Laboratory analyses

3.1.8.1 Myeloperoxidase enzyme linked immunosorbent assay (ELISA)

The myeloperoxidase (MPO) ELISA kit (Immundiagnostik, Germany) was used for the assay. Proteins were extracted from the fecal samples prior to the assay as follows: frozen fecal samples were thawed at 2-8°C and then warmed to room temperature. Up to 100mg of the thawed fecal sample was weighed on an FX-300i WP electronic weighing scale (A&D Company Ltd, UK) and the weight was recorded. For fecal samples that were less than 100mg, any amount of fecal sample available (but not less than 50mg) was weighed and recorded. One ml of

1× wash buffer was added to the weighed fecal sample and the mix was homogenized at 1400rpm for 2 minutes using a Stuart Vortex Mixer SA8 (Stuart Equipment, UK). The homogenized sample was centrifuged at 800×g for 10 minutes and the resulting supernatant was centrifuged again at 15000×g for 5 minutes using a bench-top microcentrifuge (Eppendorf, UK). The supernatant obtained from the second centrifugation was then diluted 1/50 with 1× wash buffer.

One hundred µl of the final dilution of samples above, assay controls and standards were added to corresponding ELISA plate wells. All samples were assayed in singles except assay standards and controls, which were assayed in duplicate. Each run included 5 standards of known concentration and 2 controls (low and high). Plates were then incubated for 1 hour at room temperature to allow the MPO to bind the antibodies, which were immobilized to the surface of the microtiter plate. After the incubation, the contents of the ELISA plates were decanted, and each plate well was washed 5 times with 250µl of 1× wash buffer to remove all unbound substances. This was followed by the addition of a peroxidase-labeled antibody against MPO to each well, incubation at room temperature for 1 hour and then additional washing (as above) to remove unbound substances. After the second washing step, tetramethylbenzidine (TMB), an enzyme substrate, was added to each well and plates were incubated for 15 minutes. Stop solution (1M H_2SO_4) was then added to stop the reaction and the intensity of the colorimetric reaction was determined immediately using a HALO LED96 microplate reader (Dynamica Scientific Ltd, UK) set to a wavelength of 450nm and a correction wavelength set to 620nm.

3.1.8.2 Alpha-1 antitrypsin ELISA

The alpha-1 antitrypsin (AAT) ELISA kit (Immundiagnostik) was used for the assay. Proteins were extracted from the fecal samples prior to the assay using the provided extraction buffer as follows: frozen fecal samples were thawed at 2-8°C and warmed to room temperature. From the thawed fecal sample, 15mg was weighed on an FX-300i WP electronic weighing scale (A&D Company Ltd) and the weight for each sample was recorded. For fecal samples that were less than 15mg, any amount of fecal sample available was weighed and recorded. One

and a half ml of 1× AAT extraction buffer was added to the weighed fecal sample and the mix was vortexed until suspension was homogenous. More solid samples were soaked in the buffer for 10 minutes to improve suspension before vortexing. The homogenized sample was then left to stand for 10 minutes at room temperature until any sediment settled. The resulting supernatant was diluted further with 1× wash buffer to a final dilution of 1/25,000, which was used for the assay.

The ELISA plate wells were washed 5 times with 1× wash buffer after which 100µl of diluted samples, assay controls (low and high) and 5 standards of known concentration were added to respective ELISA plate wells. This was followed by 1-hour incubation at room temperature after which the contents of the ELISA plates were decanted and each well washed 5 times with 250µl of 1× wash buffer. Thereafter, a peroxidase-labeled antibody against AAT was added to each well and plates were incubated at room temperature for 1 hour followed by another wash as previously described. After the third washing step, TMB was added to each well and incubated for 15 minutes after which the stop solution was added. The intensity of the colorimetric reaction was determined as described in section 3.1.8.1.

3.1.8.3 Neopterin ELISA

The ELISA assay was performed using the Neopterin (NEO) ELISA kit (GenWay, Biotech, San Diego, CA, USA). Fecal samples were prepared for as follows: frozen fecal samples were thawed at 2-8°C and warmed to room temperature before analysis. All samples were kept under a cardboard box on the bench as NEO is light sensitive. A maximum of 50 mg of the thawed fecal sample was weighed on an FX-300i WP electronic weighing scale and the weight for each sample was recorded. For fecal samples that were less than 50mg, any amount of fecal sample available was weighed and recorded. One ml of 1× phosphate buffered saline (PBS) (Fisher Bioreagents, USA) was added to the weighed fecal sample and the mix was homogenized on a PowerLyzer 24 Homogenizer (Qiagen, Germany) at a speed of 500rpm for 5 minutes. After the homogenization, the sample was centrifuged for 5 minutes at 800×g in a bench-

top microcentrifuge. The resulting supernatant was diluted a further 1/5 with 1× PBS and this dilution was used for the ELISA assay.

Twenty µl of samples, assay controls (low and high) and 6 standards of known concentration were added to respective ELISA wells followed by 100µl of enzyme conjugate and 50µl of Neopterin antiserum. Plates were then incubated for 1.5 hours at room temperature on a standard analog rocker (VWR, USA) at a speed of 500 rpm in the dark. After the incubation, the contents of the wells were decanted, and the wells were washed three times with 1× wash buffer. Thereafter, TMB was added to each well followed by incubation for 10 minutes at room temperature in the dark. After TMB incubation, 1M H_2SO_4 was added to stop the reaction and the intensity of the colorimetric reaction was determined as described in section 3.1.8.1.

3.1.8.4 Extraction of total genomic DNA from fecal samples

Total, genomic DNA was isolated from fecal samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc, Carlsbad, CA, now a part of Qiagen, Germany). Briefly, 250mg of fecal sample was weighed and added to a tube with PowerSoil beads. This was gently vortexed to mix and subsequently lysis buffer was added. The mix was then homogenized using the PowerLyzer 24 Homogenizer at a speed of 1500rpm for 2.5 minutes. This was followed by centrifugation at 10,000×g for 30 seconds at room temperature using a bench-top microcentrifuge. The resulting supernatant was transferred to a 1.5ml sterile tube followed by incubation with precipitation buffer at 4°C for 5 minutes and centrifugation at 10,000×g for 1 minute. The incubation and centrifugation steps above were then repeated.

The resulting supernatant was transferred to a 1.5ml sterile tube followed by the addition of a high concentration salt solution, enabling DNA, but not non-DNA organic and inorganic material, to bind to the silica membrane on a spin column filter. This mixture was loaded onto the spin column filter and centrifuged at 10,000×g for 1 minute. An ethanol-based wash solution was then added onto the spin column filter, to remove residual salt, humic acid, and other contaminants, and the spin column filter was then centrifuged at 10,000×g for 1 minute. The spin

column filter was then placed in a clean tube followed by addition of 100µl elution buffer and a 30 second centrifugation at 10,000×g.

As a quality control measure, 10% of samples were randomly selected to quantify the DNA yield using the Qubit dsDNA HS assay kit (Invitrogen, CA, USA) as follows. Five µl of the DNA sample was added to 195µl of working solution prepared by adding 1µl of the reagent to 199µl of buffer provided in the assay kit. The mix was then incubated for 2 minutes at room temperature, followed by DNA quantitation on the Qubit 2.0 fluorometer (Invitrogen). The fluorometer was calibrated with two standards (low and high concentration) before measuring the samples.

3.1.8.5 Microbial detection qPCR

Endpoint qPCR was performed to detect the presence of the following bacteria in DNA extracts from fecal samples: *Akkermensia muciniphila, Bifidobacterium longum Dorea formicigenerans,* and *Faecalibacterium prausnitzii.* To negate any unwanted effects of high DNA yield e.g. PCR inhibition and non-specific binding, the DNA sample was first diluted 1/10 with PCR grade water (Qiagen) and the diluted DNA was then used as a template in the PCR reaction. The qPCR was performed using the Rotorgene Q PCR machine (Qiagen, Hilden, Germany) where samples were run in a 72-well rotor format. The total volume of a single PCR reaction was 12.5µl and each reaction contained 6.25µl of commercially available microbial qPCR master mix (Qiagen, Germany), 0.5µl of commercially available microbial DNA qPCR assay containing primers and probes (Qiagen) and 3µl template DNA in an aqueous solution. A no template control and a microbial DNA positive control (Qiagen) were included on each run. Thermal cycling conditions for the assay were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 2 minutes.

3.1.8.6 Bacterial quantification qPCR

The published BactQuant assay[136] was used to measure total bacterial load in the genomic DNA extracted from fecal samples. Minor adjustments were made to the original protocol, which included, running the standard curve and the no-template control in singles to reduce reagent costs, reducing the number of cycles

to 35, increasing template volume from 1µl to 2.5µl and adding 2.5µl of PCR grade water to have a final reaction volume of 12.5µl. The DNA sample was diluted 1/10 prior to the assay to negate any undesirable effects of high DNA yield.

The total volume of a single PCR reaction was 12.5µl. Each PCR reaction included 1.8µM of each forward (5′-CCTACGGGDGGCWGCA-3′) and reverse primer (5′- GGACTACHVGGGTMTCTAATC-3′), 4.5µM of the TaqMan® probe (6FAM) 5′-CAGCAGCCGCGGTA-3′ (MGBNFQ), 5µl of 2×Platinum® Quantitative PCR SuperMix-UDG with ROX (Invitrogen Corp., UK) and 2.5µl template DNA. Each run included serial dilutions of PCR products of known-concentration (0.016ng/µl–1.6e-7ng/µl in 10-fold serial dilutions) and a no template control. The PCR was performed on the Rotorgene Q platform (Qiagen) with 72-well rotor format using the following thermal cycling conditions: 3 min at 50°C for UNG treatment, 10 minutes at 95°C and 35 cycles of 95°C for 15 seconds and 60°C for 1 minute.

3.1.8.7 16S rRNA gene sequencing

The 16S rRNA gene sequencing procedure included DNA library preparation, dilution and denaturation of the DNA library, paired-end DNA sequencing and preparation of 16S rRNA gene sequence reads for analysis. The flow of the V4-16S rRNA sequencing process is illustrated in **Figure 3.1.8.7.1** below.

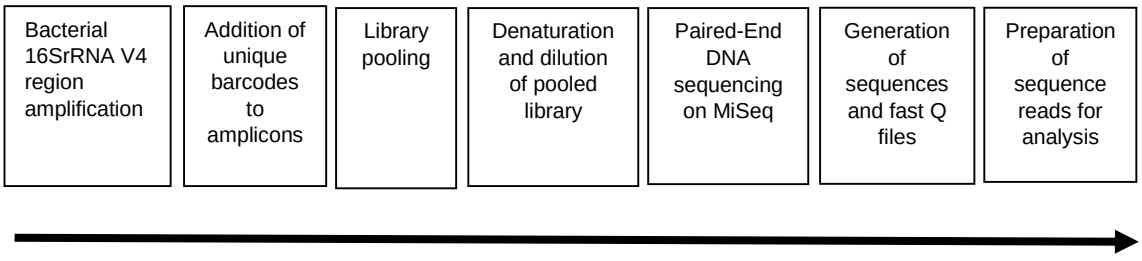

Figure 3.1.8.7.1 16S rRNA gene sequencing workflow

3.1.8.7.1 DNA library preparation

Total, genomic DNA, extracted as described in section 3.1.8.4, was used for DNA library preparation. This involved a series of PCR *i.e.* amplicon PCR and indexing

PCR. DNA libraries were prepared by amplifying an approximately 390-bp V4 region of the 16S rRNA gene during amplicon PCR using 10µM each of forward (515F_Indexed; 5'-adapter- GTGCCAGCMGCCGCGGTAA-3') and reverse (806R_Indexed; 5'-adapter-GGACTACHVGGGTWTCTAAT-3') primers, which amplify sequences for both Bacteria and Archea. Each 23.5µl reaction contained 10µl 2.5× Quantabio 5prime HotMasterMix (Quantabio, Beverly, MA, USA), 10µM primers (SIGMA, UK), 4µl template DNA and 8µl PCR grade water (Qiagen). PCR reactions were added in a 96 well-plate and amplified on a Veriti™ 96 well thermal cycler (Applied Biosystems, UK) using the following thermal cycling conditions: 94°C for 3 minutes, 35 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 45 seconds at 72°C, then 10 minutes at 72°C. Each PCR run included a mock bacterial community, which acted as a positive control, and a no template control. The mock bacterial community was composed of *Hemophilus influenza, Moraxella catarrhalis* and *Staphylococcus epidermis*. After the amplicon PCR, 4µl of amplicon was run on a 2% agarose gel stained with Ethidium Bromide (EtBr) to verify amplicon size and PCR efficiency. The agarose gel was prepared by adding 3g of agarose to 150ml of 1×Tris-Borate-EDTA (TBE) buffer. The mix was heated in a microwave until mixture was clear. This was followed by adding 7.5µl of EtBr (10mg/ml) to the mixture, which was cooled down before being poured in the gel tray. Amplicons that were checked on the agarose gel included the mock control, the no template control and a random selection of 6% of samples. Using the V4 515F-806R primers, the expected amplicon size was approximately 390bp.

Having verified amplicon sizes and PCR efficiency, all amplicons in the 96 well plate were purified using 0.6 v/v AMPure XP beads (Beckman Coulter, CA, USA) and 70% ethanol as follows. The AMPure XP beads were equilibrated to room temperature before use and then vortexed gently for 30 seconds before adding 20µl to each reaction well. The 96-well plate with the bead-amplicon mix was incubated at room temperature for 5 minutes and then placed on a super magnet plate for 2 minutes to separate the beads from solution. The cleared solution from each well was aspirated without disturbing the beads and the plates were then washed twice with 200µl of 75% ethanol, with each wash followed by incubation for 30 seconds at room temperature. The reaction plate was left to air-dry for 2 minutes and then 27.5µl of nuclease free water was added to each well of the

reaction plate and mixed by pipetting several times to fully re-suspend the beads. Following 2-minutes incubation, the plate was returned to the super magnet plate for 1 minute to separate the beads. Twenty-two µl eluate containing the amplicons was transferred to a new reaction plate, sealed and stored at -20°C to use for index PCR.

The second PCR (index PCR) was done to add unique barcodes to each DNA amplicon obtained in the amplicon PCR to enable downstream multiplexing. The sequences for the unique barcode primers were recorded on a mapping file matching each sample identifier. The total volume for a single reaction of index PCR was 49µl. Each 49µl PCR reaction contained 20µl 2.5× Quantabio 5prime HotMasterMix (Quantabio, Beverly, MA, USA), 2µM MID Illumina primers (Illumina, CA, USA), 22µl of amplicon and 5.5µl of PCR grade water (Qiagen). The index PCR was setup in a 96-well plate using the following thermal cycling conditions: 94°C for 3 minutes followed by 5 cycles of 10 seconds at 94°C, 30 seconds at 58°C and 45 seconds at 72°C then 10 minutes at 72°C. Each run included one mock control and a no template control.

After the PCR, 10µl of the amplicon was run on a 2% agarose gel to verify amplicon size and PCR efficiency. The resulting amplicons were again cleaned as described in this section above, however, twice the volume of the AMPure XP beads was used. Additionally, re-suspension of beads in index PCR was done with 15µl of elution buffer (Qiagen, Germany) instead of nuclease free water. The eluate containing the amplicons was then transferred to a new 96-well plate and each amplicon was quantified on the Qubit 2.0 Fluorometer (Invitrogen) as described in section 3.1.8.4.

3.1.8.7.2 Pooling amplicons

Before pooling individual DNA amplicons, the concentrations of individual amplicons were converted from ng/µl to nM using formula **A** below. Each amplicon was diluted to a final concentration of 12.5nM using 10mM Tris pH 8.5 and 5µl aliquots of diluted DNA from each amplicon were then mixed in one sterile Eppendorf tube. Up to 117 individual amplicons were pooled for a single sequencing run. The final pooled DNA library was quantified on a Qubit 2.0 fluorometer (as described in section 3.1.8.4) and run on the Agilent Bioanalyzer

2100 with Agilent 1000 DNA chips (Agilent Technologies Inc., USA), using a standard protocol[137], to determine its concentration and size.

$$\mathbf{A} \qquad \frac{\text{(Individual library concentration in ng/µl)}}{660\text{g/mol x 427 (average library size in bp)}} \times 10^6 = \text{XnM}$$

3.1.8.7.3 Denaturing and diluting the pooled DNA libraries for sequencing

The MiSeq v3 reagent kit (Illumina Inc., San Diego, CA, USA) was used to prepare the DNA library for cluster generation and sequencing. The MiSeq reagent cartridge was thawed in cold water for 1 hour and thereafter placed in a refrigerator (2-8°C) as the DNA library and PhiX control were being denatured and diluted. All other reagents were thawed on ice.

The DNA library was denatured with a sodium hydroxide solution (0.2N NaOH) and then diluted with hybridization buffer (HT1) to a final loading concentration of 15pM. The diluted library was spiked with 10% PhiX control, which served as an internal control for low-diversity libraries. The Phix-spiked DNA library was heat-denatured at 96°C for 2 minutes and then 600µl was loaded into the sample well of the MiSeq reagent cartridge. The MiSeq flow-cell was rinsed with Milli-Q® water and 75% ethanol then gently dried with Kimwipes™ (Kimberly-Clark™Professional, USA) before being placed into the MiSeq sequencer (Illumina, San Diego, CA, USA). The reagent cartridge was then assayed by 2×300bp paired-end sequencing for a total of 600 cycles using a standard protocol[138].

3.1.8.7.4 Preparation of the 16S rRNA sequence reads for analysis

Raw sequence reads generated by the Illumina MiSeq sequencer were automatically uploaded to BaseSpace (Illumina) where FastQ files were generated. The FastQ files were processed in QIIME 2[139] to link sequences to their individual samples, filter out poor quality sequencing reads, trim sequencing adapters and barcodes, combine each set of paired-end reads into a single sequence and cluster near-identical sequences using an identity cut-off to form operational taxonomic units (OTU). These steps are explained in detail below:

3.1.8.7.4.1 Demultiplexing

This step assigned each sequence read to the individual sample it originated from. The sequences were linked back to their original samples via the DNA barcode incorporated into each sequence during the indexing PCR. The barcode sequences, unique and corresponding to each sample, were recorded on a mapping file and using the *demux* function in QIIME 2, the sequences were assigned back to their original samples.

3.1.8.7.4.2 Quality filtering

During this step, sequencing reads were trimmed to a consistent length and low-quality reads were removed based on Phred Q scores, which is a probability of error in base calling during sequencing. High-quality score implied that a base call was more reliable and less likely to be incorrect. Thus, given an assertion, A, the quality score, Q(A), expresses the probability that A is not true, P(~A), according to the relationship:

$$Q(A) = -10 \log_{10}(P(\sim A))$$

where *P(~A)* is the estimated probability of an assertion A being wrong[140]. The relationship between the quality score and error probability is shown in **Table 3.1.8.7.4.2.1** below. Using QIIME 2, per base quality scores across all reads were manually inspected to identify where the median quality score dropped below Phred score Q=20 for forward and reverse reads respectively. Reads were trimmed at the identified threshold and filtered using the DADA2 plugin with default parameters. Trimming and filtering was performed on paired reads jointly, *i.e.* both reads had to pass the filter for the pair to pass. Additionally, the filtering steps incorporated checking for and removing chimeras.

Table 3.1.8.7.4.2.1 Relationship between the quality score and error probability

Quality Score, Q (A)	Error probability, P (~A)
10	0.1
20	0.01
30	0.001

3.1.8.7.4.3 OTU clustering and taxonomy assignment

Paired filtered reads were then merged and clustered *de novo* according to their intrinsic similarities[141]. The clustered sequences were deemed to have come from the same bacterial taxon if they were ≥ 97% identical. These clusters formed OTUs that were assigned taxonomy using a naïve Bayes classifier pre-trained on the SILVA 16S database[142]. An OTU count table with taxonomy ranking down to the genus level was created after taxonomy assignment and exported from QIIME 2 for analyses.

3.1.8.7.4.4 OTU data quality filtering and normalization

Further quality filtering was conducted on the OTU count data (generated as described in section 3.1.8.7.4.3 above) to remove spurious OTUs from the OTU table. Briefly, samples with >1000 total reads were retained and from these, only bacterial OTUs identified to the genus level, with sequences more than 0.005% of the total number of sequences[143] and a frequency of more than 0.01% in any sample were kept. To account for unequal DNA library sizes, OTU data was rarefied to 1000 reads with 1000 permutations before calculating diversity indices and determining bacterial community differences between samples or by cumulative sum scaling (CSS) before performing univariate analysis of individual taxa[144,145].

3.1.9 Statistical analyses

3.1.9.1 Primary analysis

All statistical analyses were completed in STATA 15 (StataCorp, College Station, USA) and R studio version 1.1.447[146]. Participants enrolled in the MORDOR-morbidity study who were included in the analyses of this thesis are shown in **Figure 3.1.3.2**. The primary outcomes for this thesis were child stunting, fecal microbiota diversity and composition, intestinal bacterial carriage, fecal biomarkers of EED (AAT, MPO, NEO) and the composite EED score, which were defined/calculated as detailed below.

3.1.9.1.1 Stunting

The WHO 2006 Child Growth Standards were used for age-and-sex standardization of length/height *i.e.* calculation of HAZ scores[34]. Participants with out-of-range HAZ (>6 or <-6) were not included in analyses of HAZ. HAZ scores were used in analyses as continuous variables or categorized into stunted or not-stunted centered around a HAZ score of -2. Thus, individuals with a HAZ score of <-2 were categorized as stunted while individuals with a HAZ score of ≥-2 were categorized as not stunted.

3.1.9.1.2 Fecal biomarkers of EED

A four-parameter logistic regression model (4plm) was used to calculate concentrations from optical density (OD) values generated by the ELISA assays described above (sections 3.1.8.1-3.1.8.3). The 4plm model estimates 4 parameters needed to fit a sigmoid curve namely: **a**=minimum value that can be obtained, **d**=maximum value that can be obtained, **c**=point on the sigmoid curve halfway between **a** and **d,** and **b**=steepness of curve at point **c** (see formula **B** below)**.** A sigmoid curve was fit on all OD values to determine concentrations using formula **B** below. In the formula below, **a, b, c** and **d** are the 4 parameters estimated by the 4plm whereas ***y*** is the absorbance value and ***x*** the unknown concentration.

B

$$x = c\left(\frac{a-d}{y-d} - 1\right)^{\frac{1}{b}}$$

Samples with concentrations above the highest standard were diluted further and re-tested. Samples with concentrations below the lowest standard were diluted less and re-tested. If they were still low after being re-tested, they were assigned a concentration value of 0.001. The magnitude of intestinal inflammation was determined from the MPO or NEO whilst the AAT concentrations were used to determine the magnitude of intestinal permeability. Additionally, associations between intestinal inflammation or permeability and stunting or HAZ scores were explored using regression models.

The three biomarkers (MPO, AAT and NEO) were combined to form an EED score as described by Kosek *et al.*[53] with slight modifications. Briefly, raw concentrations for each biomarker were categorized into quartiles according to

their distribution as follows: 0 (concentration less than or equal to the 25th quartile), 1 (concentration between the 25th and 75th quartile) and 2 (concentration more than or equal to 75th quartile). Principal components with natural log of raw continuous data were generated for each biomarker. Instead of using rounded weightings from the principal component analysis (PCA) to multiply with the quartile-categorized values as stated in the published method[53], absolute weightings were used to generate continuous EED scores. The products of the three biomarkers were then summed to get the EED score. The steps taken for the formation of the composite EED score are summarized in **Figure 3.1.9.1.2.1** below. Associations between the composite EED score and stunting were also explored using regression models.

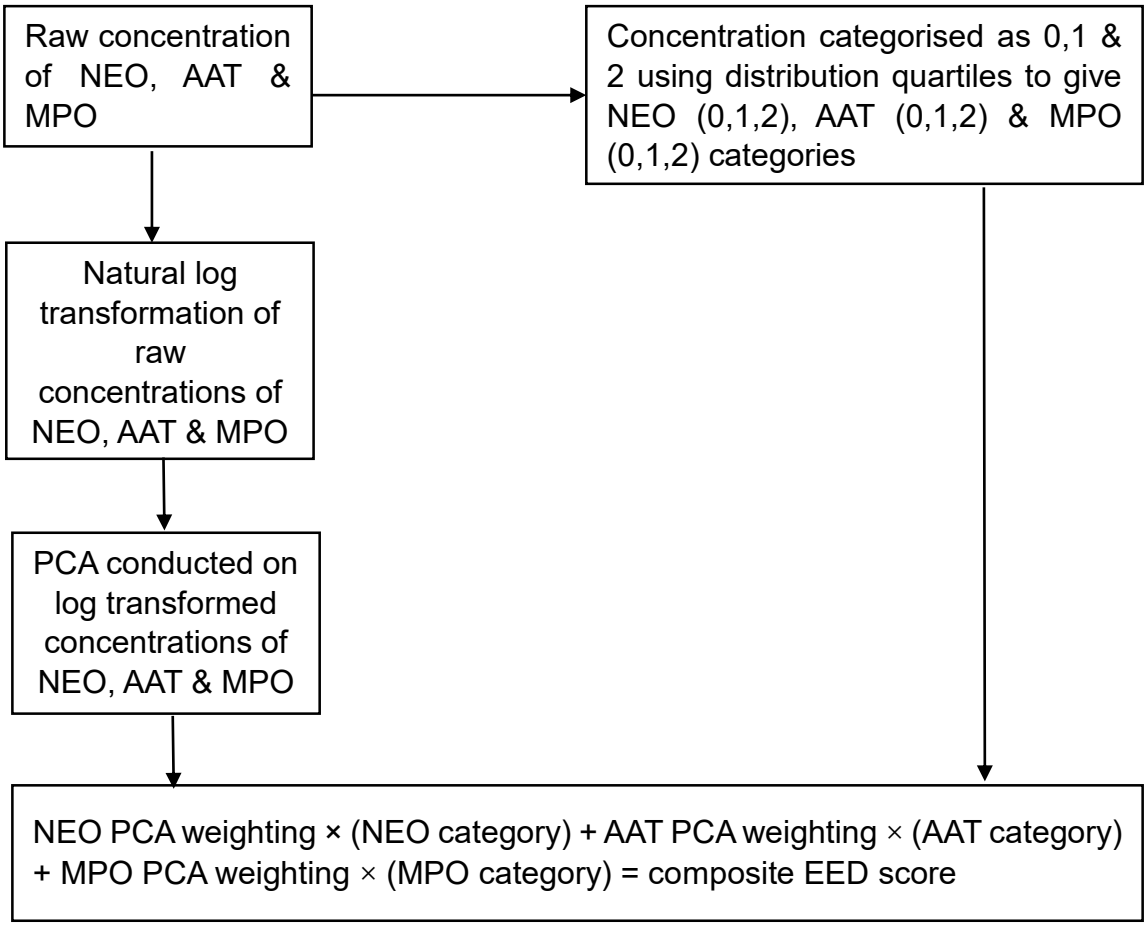

Figure 3.1.9.1.2.1 Steps for forming the composite EED score

3.1.9.1.3 *Intestinal carriage*

Published biostatistical rules on classification of continuous real-time qPCR data into positive or negative[147] were adopted and used to classify the microbial qPCR results as positive or negative with minor modifications. Briefly, a 4plm was fitted

on raw amplification data and its parameters were used to estimate fluorescence values through each qPCR cycle for each sample. The estimated fluorescence values were then compared to the actual fluorescence values to determine the variation between the two values. Additionally, the final actual fluorescence values for each sample were modelled onto mixtures of two normal distributions. A sample was considered positive if the percentage variation between its estimated and actual fluorescence values was less than 15 and if the sample clustered within the right-most population of a mixtures-model of two normal distributions (final fluorescence >0.15). The steps for the determination of a positive or negative qPCR result are summarized in **Figure 3.1.9.1.3.1** below. Prevalence of bacterial carriage was then calculated from the qPCR results. Additionally, associations between intestinal bacterial carriage and fecal biomarkers of EED (individually or as a composite EED score) were explored using logistic regression analysis.

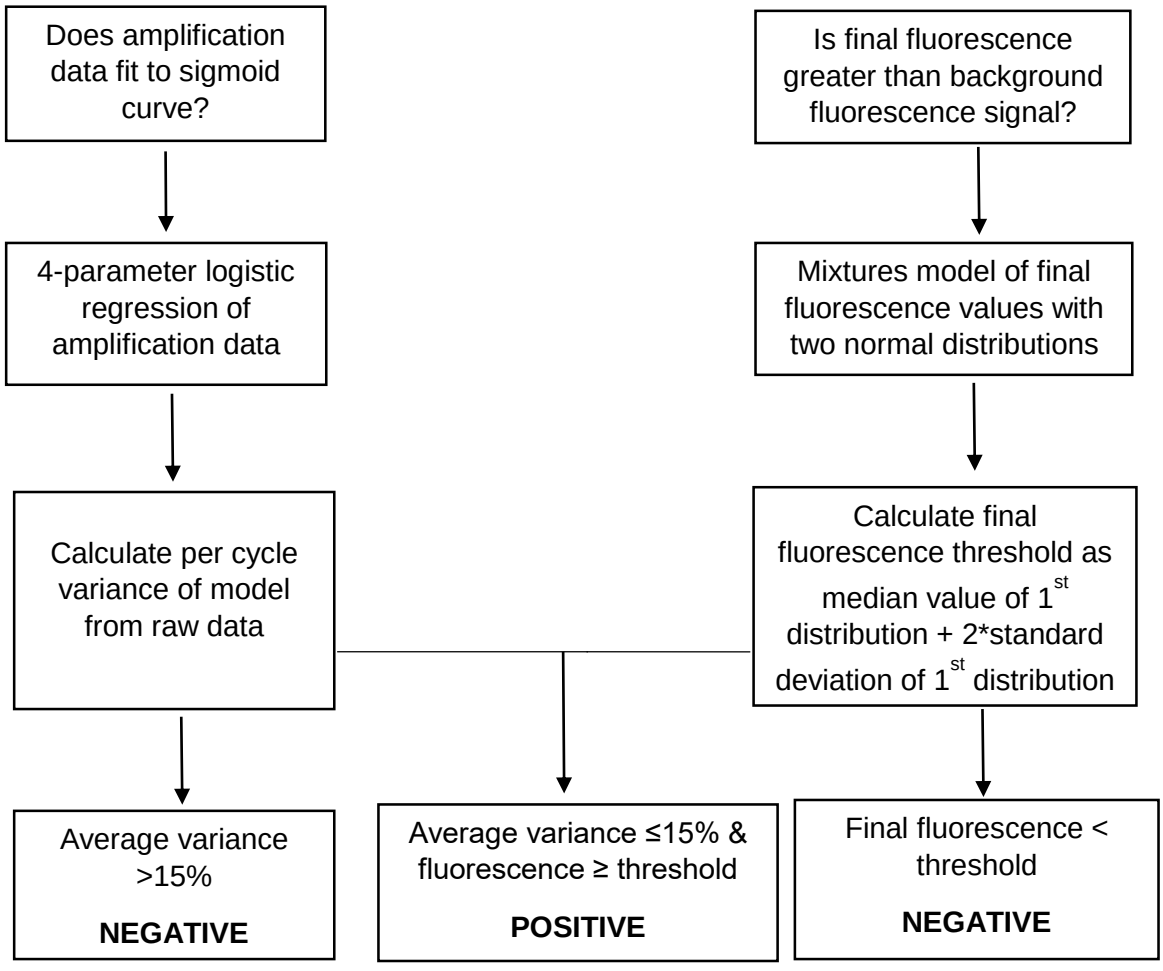

Figure 3.1.9.1.3.1 Steps for determining a positive or negative qPCR result

3.1.9.1.4 Gut microbiota diversity and composition

Shannon (H) and Simpson (D) diversity indices were calculated from the OTUs to indicate alpha diversity in the fecal samples using the phyloseq R package[148]. Shannon index considers both richness and evenness whereas Simpson index considers evenness of OTUs in each sample. A larger Shannon or Simpson value indicates a higher level of diversity. To determine bacterial phylogenetic distances between samples, weighted and unweighted UniFrac distance matrices were calculated using the phyloseq R package. The UniFrac distance metric compared relatedness of community members based on phylogenetic distances between organisms; the weighted UniFrac metric indicated presence or absence of OTUs while accounting for relative abundance whereas unweighted UniFrac only indicated presence or absence of OTUs. The phylogenies used to calculate the UniFrac distances were computed using RAxML v8.2.11[149] from a variable sites alignment using a generalized time-reversible (GTR) + gamma model.

Differences in the distribution of continuous variables between groups were calculated using Student t-test or ANOVA for parametric data while the Wilcoxon rank sum test or the Kruskal-Wallis test was used for non-parametric data. Linear regression models were used to assess the relationship between predictors and continuous outcomes while logistic regression models were used to assess associations between the predictors and dichotomous outcomes. Both models were adjusted for a set of covariates. To compare bacteria community compositional differences between groups, PERMANOVA with 1000 permutation tests using the default Bonferroni *P*-value correction in Adonis function, under the vegan R package[150], was run on UniFrac distance matrices. Non-normalized OTU data was used to determine differential abundance of OTUs between samples using the DESeq2 package in R[151]. Statistical significance of $\log_2$ fold changes was assessed using the default Wald test with Benjamini-Hochberg p-value correction in DESeq2. Cut-off for all significant tests was set at $P<0.01$. The relationship between the identified differentially abundant OTUs and outcomes of interest was tested using a zero-inflated negative binomial regression model,

implemented within the glmmTMB R package[152], to adjust for probability of zero count in relation to library size controlling for a set of covariates.

3.1.9.2 Secondary analysis

The secondary analyses for this thesis included testing for associations between demographics (age, sex, geographic zone) and biomarkers of EED, gut microbiota diversity and composition and intestinal carriage. Relative abundance of the selected bacteria (*Akkermensia muciniphila, Bifidobacterium longum Dorea formicigenerans,* and *Faecalibacterium prausnitzii*) in the fecal samples was also calculated using the delta C_T method[153]. For this, amplification data for each of the 4 bacteria was normalized relative to the amplification of the bacterial 16S rRNA gene (described in section 3.1.8.6) in the same sample. The bacterial 16S rRNA gene was chosen as most suitable for normalization as it is expressed in all bacteria. Associations between predictors (intestinal bacterial carriage, relative bacterial abundance, fecal biomarkers of EED) and weight metrics were also explored. The weight metrics included weight-for-age (WAZ) and weight-for-height (WHZ) Z scores, which were calculated using the WHO 2006 Child Growth Standards[34]. Participants with out-of-range WAZ (>5 or <-6) and WHZ scores (>5 or <-5) were not included in analyses of WAZ and WHZ respectively. WAZ and WHZ were used as continuous response variables or categorized into underweight or not-underweight and wasted or not-wasted as shown in **Table 3.1.9.2.1** below.

Table 3.1.9.2.1 Categorization of WAZ and WHZ scores into growth status

Z score	Cut-off	Category
WAZ	<-2	Underweight
WAZ	≥-2	Not underweight
WHZ	<-2	Wasted
WHZ	≥-2	Not wasted

3.1.10 Data management

Demographic (date of birth, sex and location), treatment and anthropometry data (weight and height) from the MORDOR trial were captured electronically in the

field on tablets using the Salesforce platform (Salesforce.com, Inc., San Francisco, CA, USA), which uploaded the data to a password secured central server at the end of each day. The server was only accessible to MORDOR trial investigators. Laboratory data was captured on various platforms depending on the experiment. OD values for all ELISA assays were captured in the Halo LED 96 capture software and exported as comma-separated values (CSV) files. All quantitative PCR amplification data was captured in the Rotorgene Q software version 2.3.4 and later exported as CSV files. Raw 16SrRNA gene sequencing data was automatically uploaded to BaseSpace (Illumina) and FastQ files were generated at the end of each run. All biological samples were assigned de-personalized identifiers that could not be linked to participant data in the laboratory. Each participant was given a unique identifier in the field, which was used to link anthropometry, demographic, treatment and laboratory data after all experiments were complete.

To ensure quality of data collected, demographic data, including dates of birth, was verified by MORDOR trial staff who revisited selected households in the weeks following the census. For demographic data that had implausible dates of birth, MORDOR trial staff revisited participants' homes and verified dates of birth against the health passports. Anthropometry data was captured using an electronic collection tool that had pre-set acceptable ranges for anthropometry measures so that flags would appear when unlikely measures were entered. For data collected through laboratory experiments, positive and negative controls were included in every experiment. Results of the controls were compared to their expected results and runs for which the controls did not give the acceptable results were repeated.

Data was cleaned in STATA 15 and RStudio version 1.1.447 to make it suitable for analysis. All data were checked for consistency, duplicates, completeness and implausible values. Additionally, all sample and participant identification numbers were checked for clerical errors. Implausible and inconsistent anthropometry and treatment data were verified by the MORDOR trial coordinator. All data with inconsistencies that could not be resolved were excluded from analysis. Anonymized qPCR, 16S rRNA gene sequencing and ELISA data will be made publicly available to other researchers via accession in the LSHTM research data

repository. Additionally, the 16S rRNA gene sequences will be deposited in the Sequence Read Archive (SRA). However, data-linking laboratory data to named participants is restricted and as such, this data will not be made available to outside researchers.

4. Fecal biomarkers of intestinal inflammation and permeability and associations with growth outcomes in rural Malawian children

4.1.1 Introduction

EED is a subclinical condition of the gut characterized by structural changes in the intestinal epithelium and underlying chronic inflammatory responses[2,3]. More than two-thirds of children living in low-income settings are thought to have signs of EED[11,12]. The cause of EED is not well defined, however, its epidemiology suggests microbial infections and exposure to poor sanitation and hygiene play a role. A hypothetical model suggests that ingestion of contaminated food or water as well as exposure to unsanitary conditions changes the composition and function of the gut microbiota, resulting in structural changes of the intestinal epithelium. This then allows translocation of microbes or microbial antigens from the lumen into the systemic circulation causing chronic immune activation, which, in turn, causes growth faltering either by repressing growth hormones or by repartitioning nutrients away from growth towards increased immune metabolism[47].

Confirming a diagnosis of EED requires visualization of the intestinal epithelium using endoscopy or biopsy. Studies that have utilized these techniques to investigate mechanisms underlying EED development and the impact of some interventions on EED have reported important findings. A recent study, involving adults in Zambia, conducted to better understand mechanisms underlying EED reported an association between translocation of gut microbes and defects in the intestinal epithelial lining[154]. The study collected intestinal biopsies from adults who were HIV seropositive or seronegative and not infected by helminths at the time of enrollment. Morphometry of the collected biopsies showed villous blunting and crypt lengthening with all the biopsies having a Villous Height:Crypt Depth less than the normal Western ratio of 3:1[155] regardless of participant HIV status. In addition, endomicroscopy of the duodenum showed epithelial lesions and evidence of gut leakage in 80% of the participants. Immunohistochemical analysis of the epithelial tight junction integrity and distribution revealed loss of tight junctions as indicated by presence of claudin 4, a tight junction protein, at sites of cell shedding. Also, transcriptomic analyses of the biopsies showed an association between biopsies with severe and mild EED and downregulation of genes responsible for epithelial repair processes. Another study in Zambian adults showed that multiple macronutrient supplementation improved small bowel

villous height and absorptive area[156]. The study reported an increase in villous height and Villous Height:Crypt Depth ratio in HIV seronegative participants who received multiple macronutrient supplement over a period of 6 weeks compared to their counterparts who received a placebo over the same follow-up period.

Although intestinal histopathological tests provide important details that can inform interventions that may reduce EED, their use in asymptomatic young children is considered unethical as endoscopy or biopsy are invasive tests. Alternative non-invasive tests are therefore used to measure different aspects of EED in children. The urinary L:M ratio test is one of the commonly used non-invasive tests for EED and measures intestinal absorptive capacity and diminished intestinal barrier function. The test involves challenging a subject, after a period of fasting, with a solution that has a known concentration of lactulose and mannitol [19,20]. Lactulose is a large sugar that is not normally absorbed by the small intestine while mannitol is a smaller sugar that is absorbed by the small intestine in proportion to absorptive surface area. Both sugars are excreted in the urine a few hours after ingestion without being metabolized. As such, urinary mannitol measures absorptive capacity whereas urinary lactulose measures diminished barrier function. Despite its significant association with intestinal histopathological changes[3], the L:M ratio test is inconsistent among studies; procedural details like fasting prior to ingestion, sugar dosage, time of urine collection and method of detection vary among studies thereby making comparison of results between studies difficult. Additionally, there are technical difficulties associated with sample collection for the test, especially from infants, as the test requires fasting before collection of urine sample and a long duration of sample collection. Leakage of sample and contamination by stool are other additional challenges in infants.

The lack of a convenient yet robust diagnostic test for EED makes it difficult to understand the pathobiology of EED and to determine interventions likely to lead to significant reductions in EED. Potential biomarkers using stool and blood are being tested as proxies of EED[14,56,60,157-159]. These biomarkers measure the various processes involved in EED, which include intestinal epithelial damage, intestinal inflammation, intestinal permeability, microbial translocation and systemic inflammation[47], and can easily be quantified in blood and/or fecal

samples using ELISA techniques. Blood and fecal samples are relatively easy to collect from children and ELISA techniques are easy to perform, therefore the use of these biomarkers to aid EED diagnosis in children is feasible.

MPO, AAT and NEO are some of the potential biomarkers that are being evaluated for use in EED diagnosis. MPO is an enzyme found in the granules of neutrophils, which are first-line responders to acute-phase inflammation caused by bacterial infection. Inside the granules of the neutrophils, MPO catalyzes the oxidation of substances through the hydrogen peroxide (H_2O_2) system, which has a toxic effect on many microorganisms[160]. Thus, MPO is a marker of neutrophil activity and high levels of fecal MPO indicate intestinal inflammation. In serum, AAT makes up the majority of serine protease inhibitors and protects tissues from protease damage during inflammation. AAT is synthesized primarily in the liver but also to a small extent in intestinal macrophages, monocytes, and intestinal epithelial cells. Since AAT is relatively resilient against enzymatic digestion, its increased levels in fecal samples indicate intestinal protein loss and increased mucosal permeability. NEO, on the other hand, is a low molecular weight molecule belonging to the chemical group known as pteridines. It is synthesized by cellular immune reaction of macrophages and dendritic cells upon stimulation with the cytokine interferon gamma (IFN-γ) and subsequently released. Thus, elevated fecal levels of NEO indicate intestinal inflammation.

Studies looking at the association between individual concentrations of AAT, MPO, NEO and growth in children at risk of developing EED have yielded mixed results. One multi-site, longitudinal study that assessed the relationship between these 3 biomarkers and linear growth in infants living in low-income settings of South Asia, Sub-Saharan Africa and Latin America showed that high levels of AAT, MPO, NEO predicted a decline in infant's length-for-age z (LAZ) scores in the subsequent 6 months within a 15-month follow-up period[53]. Similarly, a recent case-control study examining the link between potential biomarkers of EED and subsequent growth impairment in rural Brazilian children aged between 6-26 months showed an association between higher fecal levels of MPO or AAT and growth faltering[54]. In this study, levels of fecal biomarkers of EED in malnourished and age-sex matched non-malnourished controls were measured at baseline. Follow-up anthropometry measurements were taken at 2 to 6 months after initial

sampling. The study reported a significant relationship between high levels of MPO or AAT at baseline and poor HAZ scores in the subsequent months of follow-up. Arndt *et al.* utilized data from the Bangladeshi site within the multi-site, longitudinal study described above to describe longitudinal patterns of AAT, MPO and NEO and their contribution to linear growth failure in Bangladeshi children. Child anthropometry measurements were collected every month for 2 years and AAT, MPO and NEO were quantified in fecal samples that were collected quarterly throughout the follow-up period. This study did not find any association between levels of AAT, MPO or NEO in the full follow-up period (3-21 months) and subsequent 3-month linear growth[55]. Another study in rural Bangladeshi children that investigated the relationship between biomarkers of EED and stunting in under five children also reported no significant associations between baseline levels of AAT, MPO or NEO and stunting[56]. In this study, levels of AAT, MPO, NEO and calprotectin were measured in fecal samples collected at baseline in children aged 6-30 months. Anthropometry measurements were also collected at the 9-month follow-up visit. No significant associations were observed between baseline levels of AAT, MPO or NEO and stunting at the 9-month follow-up visit.

Available data suggests that no single biomarker is sufficient to indicate EED[161,162]. In the multi-site, longitudinal study described above[53], Kosek *et al.* combined raw concentrations of AAT, MPO and NEO using PCA to form a composite EED score. They then used the composite EED score to explore the relationship between EED and linear growth in infants from low-income settings of South Asia, Latin America and Sub-Saharan Africa. The study reported an association between higher composite EED scores and a projected decline in LAZ scores in the subsequent 6 months[53]. In a different study, George and colleagues adopted Kosek *et al*.'s composite EED score and explored its relationship with growth in Bangladeshi children. This study reported that children with higher composite EED scores were more likely to be underweight compared to children with lower composite EED scores[56].

The present study investigated the prevalence and magnitude of intestinal inflammation and permeability in rural Malawian children using these biomarkers. Associations between fecal biomarkers of intestinal inflammation or permeability,

measured individually and as composite scores, and measures of growth impairment were also assessed.

4.1.2 Study design and analyses

This study utilized data and biological samples collected by the MORDOR-morbidity study, the details of which are described in chapter 3. Briefly, the baseline MORDOR-morbidity aimed to recruit 1200 randomly selected children aged between 1-59 months and resident in 5 geographical zones of Mangochi district, Malawi. One-thousand- ninety children from a randomization list of 1704 names were enrolled into the MORDOR-morbidity study at baseline. Anthropometry measurements (height and weight), demographics (age and sex) and fecal samples were collected as described in chapter 3. Height and weight measurements and demographics were subsequently used to calculate HAZ, WAZ and WHZ scores using WHO growth standards[34].

Protein extracts from the fecal samples were used to quantify fecal MPO, AAT and NEO levels using ELISA assays described in sections 3.1.8.1 to 3.1.8.3 of chapter 3. Briefly, fecal samples, collected and archived at -80ºC until processing, were diluted 1/100 (for NEO), 1/500 (for MPO) and 1/25,000 (for AAT) and then processed using commercially available kits. Determination of fecal MPO, AAT and NEO concentration from ELISA absorbance values was done using the 4plm in RStudio[146]. Fecal samples with out-of-range concentration values were re-tested at a different dilution. Both MPO and NEO indicated intestinal inflammation whereas AAT indicated intestinal permeability and protein loss.

To describe the prevalence of intestinal inflammation and permeability, proportions of fecal samples with elevated concentrations of AAT, MPO and NEO relative to Western values as reported in the literature[53,161-163] were calculated. Additionally, the distribution of biomarker concentration was calculated by age, sex and geographic location and the differences between groups were statistically tested using the Kruskal-Wallis or Wilcoxon rank sum tests. HAZ, WAZ and WHZ score distributions between normal and elevated biomarker concentration or low and high composite EED score were calculated and the differences were tested by Student's t-test. Furthermore, associations between

biomarkers, as individual markers and as a composite EED score (using a method by Kosek *et al.*[53] described in chapter 3) and stunting, wasting or underweight were also investigated.

4.2 Results

4.2.1 Demographic characteristics and descriptive statistics

The MORDOR-Malawi morbidity study enrolled 1090 children at baseline however only 709 of these chose to participate in the fecal sampling; enrollment and participation in fecal sample collection is shown in **Figure 3.1.3.2** of chapter 3. Of the 709 samples collected, 523 fecal samples were available for inclusion in this study. Characteristics of all enrolled children and those included in the final analyses are shown in **Table 4.2.1.1**. The proportion of participants residing in Makanjira zone were comparable between all enrolled participants and those included in the final analyses, however, the distribution of participants from the remaining zones was different. Average height, weight, age and sex of participants included in the analyses were different from all enrolled participants (**Table 4.2.1.1**).

Table 4.2.1.1: Characteristics of participants who were included in analyses compared to all enrolled participants.

Participant characteristic	All enrolled participants	Participants with fecal sample included in analyses	P-value
Number of participants	1090	523	
Male sex N (%)	487 (44.68%)	264 (50.5%)	0.03
Mean (SD) height, cm	83.38 (12.45)	85.02 (11.56)	0.005
Mean (SD) weight, kg	11.34 (3.12)	11.76 (2.94)	0.01
Mean (SD) age, months	29.59 (16.42)	31.97 (16.02)	0.008
Geographical location			
Chilipa N (%)	235 (21.6%)	147 (28.11%)	0.005
Makanjira N (%)	225 (20.6%)	100 (19.12%)	0.518
Mangochi N (%)	237 (21.7%)	87 (16.63%)	0.02
Monkey Bay N (%)	192 (17.6%)	126 (24.09%)	0.003
Namwera N (%)	201 (18.4%)	63 (12.05%)	0.001

4.2.2 Distribution of biomarker concentration in the fecal samples

Out of the 523 fecal samples that were included in the final analyses, 488 had ELISA assay results for MPO, 425 for NEO, 495 for AAT and 425 had results for all the 3 markers. Fecal biomarker concentration distribution was calculated in all samples and compared by age, sex and geographic location. Concentration of the three biomarkers was positively skewed (**Figures 4.2.2.1 - 4.2.2.3**). A larger proportion of children (62%) had elevated NEO levels relative to reference values (**Figure 4.2.2.1**). Approximately, one third of the children had elevated MPO levels (**Figure 4.2.2.2**). AAT was the least elevated fecal biomarker in all children; 16% of the children had elevated levels of fecal AAT (**Figure 4.2.2.3**). The relationship between the biomarkers was tested using Pearson's correlation and no relationship was found (**Table 4.2.2.1**)

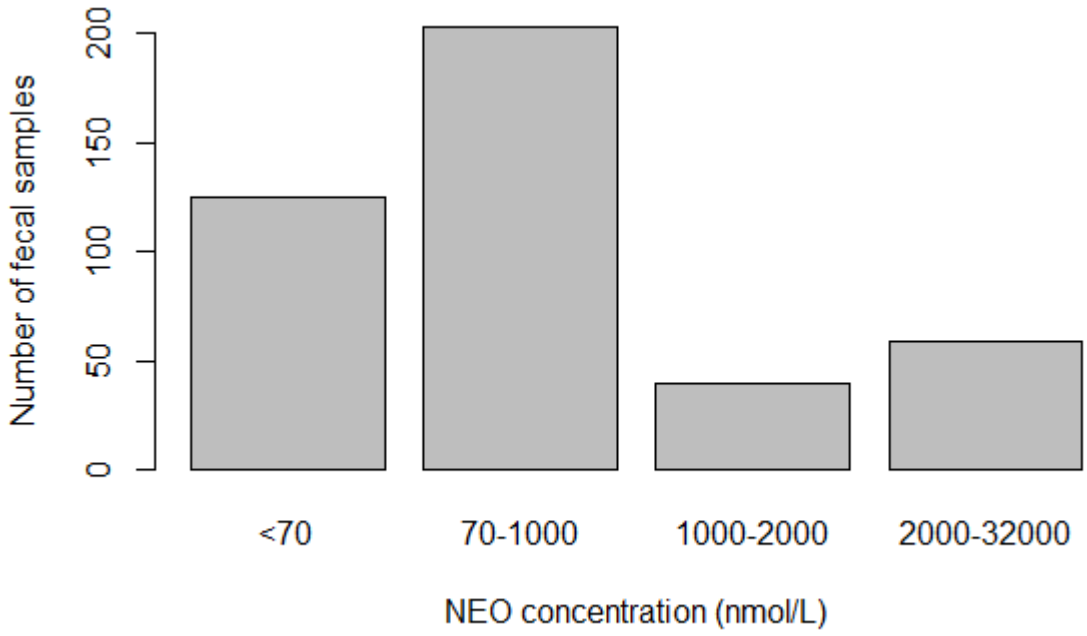

Figure 4.2.2.1: Fecal NEO concentration distribution. *Normal values were based on Western values reported in the literature[53,161,162](NEO <=70 nmol/L). 324(61.9%) fecal samples had NEO concentration of more than 70nmol.*

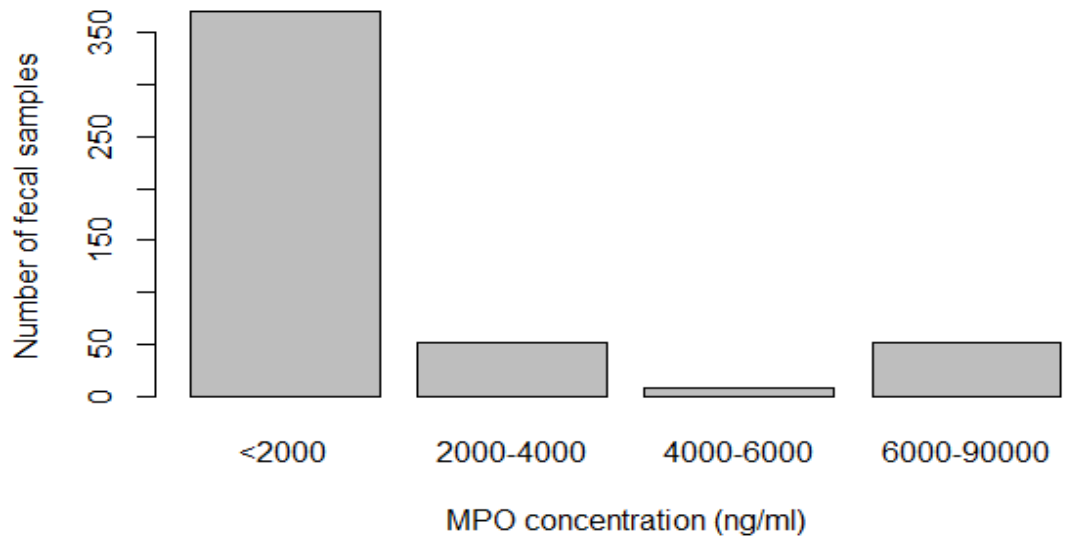

Figure 4.2.2.2: Fecal MPO concentration distribution. *Normal values were based on Western values reported in the literature[53,161,162] (MPO <=2000 ng/ml). 106 (20.3%) fecal samples had MPO concentration more than 2000ng/ml.*

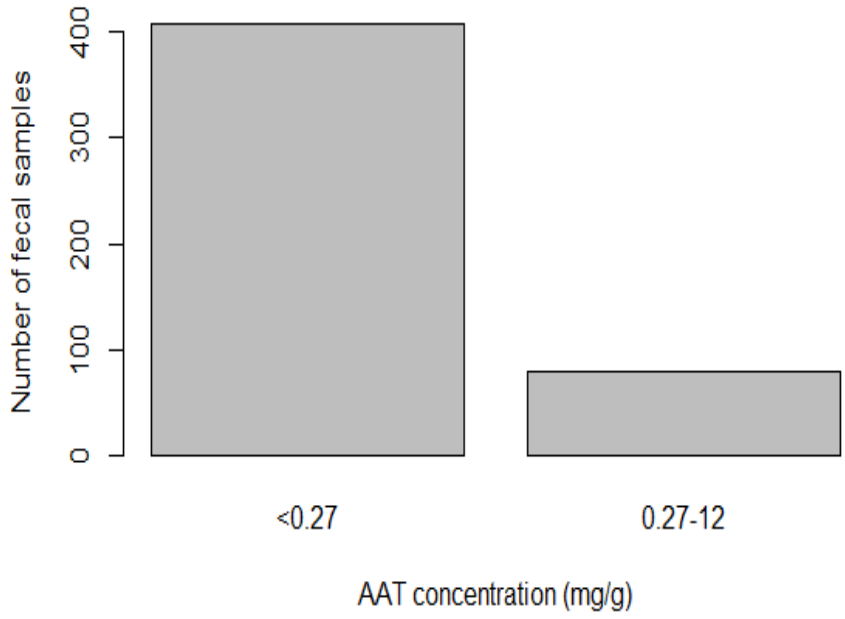

Figure 4.2.2.3: Fecal AAT concentration distribution. *Normal values were based on Western values reported in the literature[53,161,162](AAT <=0.27 mg/g). 81 (15.6%) fecal samples had AAT concentration more than 0.27mg/g.*

Table 4.2.2.1: Relationship between the individual biomarkers of EED.

Variable	[a]Coefficient (95%CI)	[a]P-value
AAT vs MPO	0.05 (-0.04,0.14)	0.24
AAT vs NEO	0.002 (-0.09,0.092)	0.97
MPO vs NEO	-0.012 (-0.11,0.08)	0.81

CI = confidence interval

[a]*The correlation coefficient (95% CI) and P-values were obtained with Pearson's product-moment correlation.*

Child age in months was then categorized into 5 groups as follows: 0-12, 13-24, 25-36, 37-48 and 49-60. The Kruskal-Wallis test was used to test for the differences in biomarker concentration distribution among the age groups. There were significant differences in biomarker distribution among the 5 age groups. Median fecal concentration for all the 3 biomarkers decreased with age (**Table 4.2.2.2**). Biomarker concentrations were also compared between the male and female sex. The Wilcoxon rank sum test did not show any differences in MPO and AAT concentrations by sex, but NEO concentration was higher in female children compared to male children (**Table 4.2.2.3**). Comparison of biomarker distribution by geographical location showed differences in the distribution of MPO and AAT. Median MPO and AAT concentrations were higher in fecal samples from children in Namwera compared to the other geographical zones while fecal samples from children in Chilipa had lower concentration of MPO (**Table 4.2.2.4**).

Table 4.2.2.2: Biomarker concentration by age

Variable	Age (months)					
	0 -12	**13 - 24**	**25 - 36**	**37 - 48**	**49 - 60**	**[a]P-value**
Number of fecal samples	68 (MPO) 55 (NEO) 66 (AAT)	104 (MPO) 99 (NEO) 105 (AAT)	86 (MPO) 85 (NEO) 90 (AAT)	114 (MPO) 108 (NEO) 117 (AAT)	86 (MPO) 81 (NEO) 85 (AAT)	
MPO (ng/ml)	4255 (1391,14556)	971 (353,2131)	580 (249,1326)	402 (218,975)	290 (173,530)	2.2e-16
NEO (nmol/L)	2101 (1050,4153)	402 (112,1491)	133 (37,332)	134 (58,406)	63 (12,165)	1.069e-14
AAT (mg/g)	0.25 (0.08,0.72)	0.1 (0.04,0.24)	0.06 (0.02,0.13)	0.06 (0.02,0.12)	0.03 (0.02,0.07)	2.2e-16

The concentrations are presented as median (25^{th},75^{th} quartile).

[a]P-value obtained from a Kruskal-Wallis test.

Table 4.2.2.3: Biomarker concentration distribution by sex.

Variable	Sex		[b]P-value
	Male	Female	
Number of stool samples	228 (MPO) 231 (AAT) 213 (NEO)	230 (MPO) 232 (AAT) 215 (NEO)	
MPO (ng/ml)	610 (229,1612)	518 (254,1883)	0.91
AAT (mg/g)	0.06 (0.02,0.16)	0.08 (0.03,0.18)	0.21
NEO (nmol/L)	140 (40,545)	201 (72,1116)	0.02

The concentrations are given as median (25^{th},75^{th} quartile).

[b]P-value obtained from a Wilcoxon rank sum test.

Table 4.2.2.4: Age and biomarker concentration distribution by geographical location.

Variable	Geographical location					
	Chilipa	**Makanjira**	**Mangochi**	**Monkey Bay**	**Namwera**	**P-value**
Number of children	147	97	77	136	58	
Mean (SD) age	31 (16.7)	32 (14.6)	32.6 (16.3)	33.7 (16.4)	30 (15.1)	0.59*
Number of fecal samples	139 (MPO) 116 (NEO) 140 (AAT)	97 (MPO) 81 (NEO) 98 (AAT)	82 (MPO) 75 (NEO) 86 (AAT)	113 (MPO) 98 (NEO) 111 (AAT)	57 (MPO) 51 (NEO) 60 (AAT)	
MPO (ng/ml)	393 (156,1170)	556 (254,1574)	707 (339,1275)	577 (256,1973)	850 (269,2117)	0.009$^{\alpha}$
NEO (nmol/L)	229 (55,1092)	117 (53,402)	161 (86,446)	124 (40,329)	356 (80,1582)	0.06 $^{\alpha}$
AAT (mg/g)	0.08 (0.04,0.22)	0.08 (0.02,0.16)	0.07 (0.03,0.2)	0.04 (0.01,0.09)	0.1 (0.05,0.25)	2.66e-7$^{\alpha}$

Biomarker concentrations are given as median (25^{th} ,75^{th} quartile).

*Denotes P-value obtained from ANOVA whereas $^{\alpha}$denotes P-value obtained from a Kruskal-Wallis test.

4.2.3 Fecal biomarkers of EED were not associated with growth outcomes

Individual biomarkers of EED (MPO, NEO and AAT) and anthropometric scores (HAZ, WAZ and WHZ) were used to investigate the association between intestinal inflammation or permeability and growth outcomes. Of all the participants (n=523) whose fecal samples were assayed for MPO, AAT or NEO, 488 had weight or height measurements and demographic (age and sex) data available. After exclusion of Z scores that were out of range (5 out-of-range HAZ, 2 out-of-range WAZ and 3 out-of-range WHZ scores), 483 participants were included in the analyses of HAZ, 486 in the analyses of WAZ and 485 in the analyses of WHZ. HAZ, WAZ and WHZ scores were used as continuous variables or categorized into stunted or not-stunted, underweight or not and wasted or not wasted respectively as described in chapter 3.1.9. HAZ, WAZ and WHZ scores were normally distributed (**Figures 4.2.3.1**). The proportion of children who were stunted was 38.9% (188/483), wasted 4.9% (24/485) and underweight 16.3% (79/486).

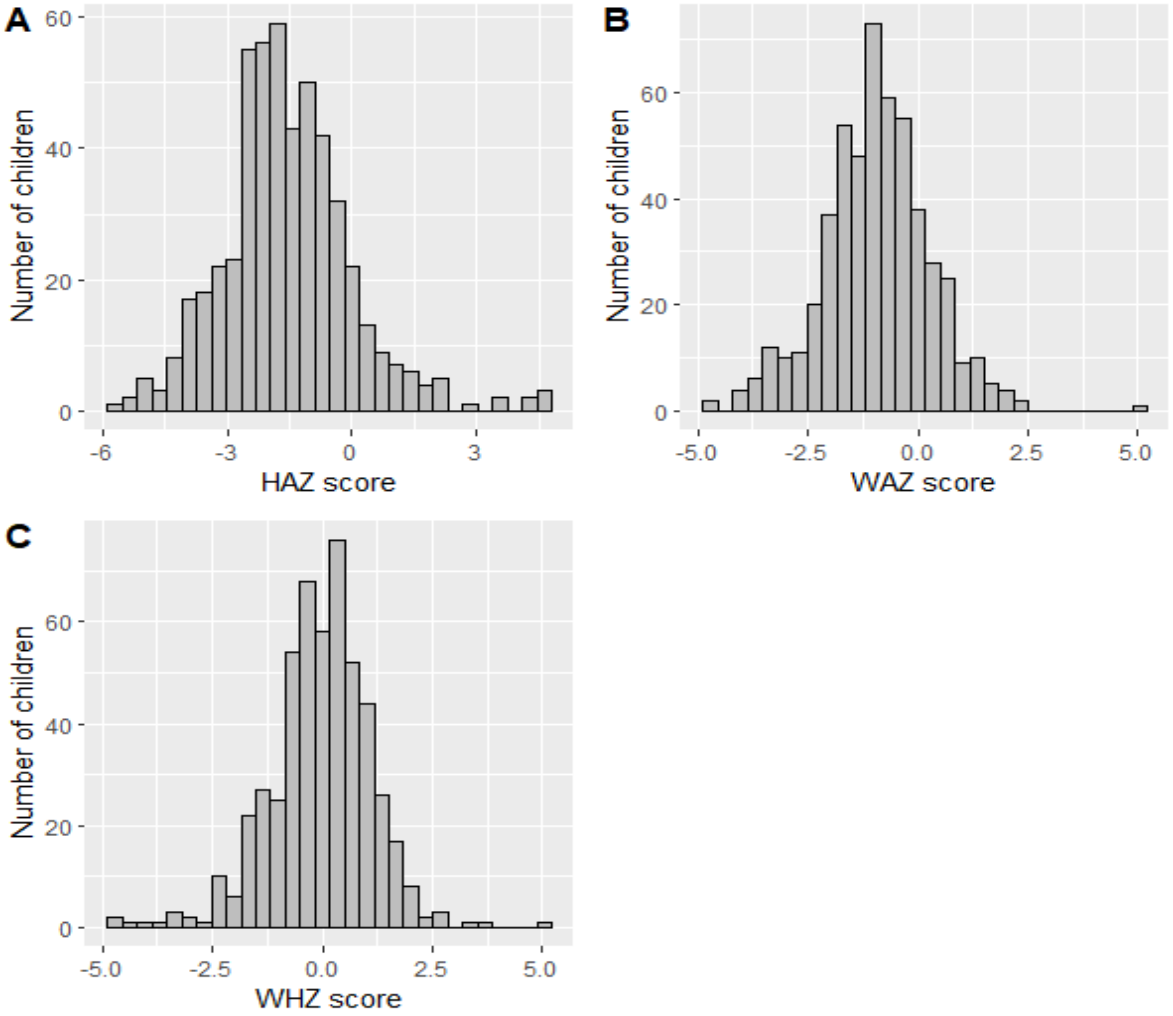

Figure 4.2.3.1 (A) HAZ, (B) WAZ and (C) WHZ score distribution for all the participants included in the final analyses. *Mean (SD) HAZ score was -1.54 (1.54), WAZ score was -0.91 (1.21) and WHZ score was -0.05 (1.17).*

Raw concentrations of NEO, AAT and MPO were categorized as normal or elevated relative to the reference values; biomarker concentrations less than or equal to the reference value were categorized as normal while concentrations higher than the reference value were categorized as elevated. HAZ, WAZ and WHZ score distribution between normal and elevated biomarker concentration was not different in any individual biomarker (**Table 4.2.3.1**). There was no association between each of the biomarkers and stunting or wasting, however, normal NEO concentration tended to have an association with reduced chances of being underweight (**Table 4.2.3.2**).

Table 4.2.3.1 HAZ, WAZ and WHZ distribution in children with normal and elevated biomarkers.

	NEO			MPO			AAT		
Variable	Normal	Elevated	P-value	Normal	Elevated	P-value	Normal	Elevated	P-value
Mean (SD) HAZ	-1.69 (1.4)	-1.47 (1.6)	0.22	-1.63 (1.49)	-1.31 (1.68)	0.08	-1.62 (1.49)	-1.4 (1.88)	0.32
Mean (SD) WAZ	-1.03 (0.97)	-0.91 (1.27)	0.28	-0.92 (1.15)	-0.83 (1.3)	0.37	-0.96 (1.2)	-0.82 (1.3)	0.37
Mean (SD) WHZ	-0.06 (0.94)	-0.05 (1.26)	0.91	0.002 (1.08)	-0.11 (1.24)	0.38	-0.05 (1.08)	-0.04 (1.45)	0.92

P-values obtained with Student's t-test.

Table 4.2.3.2 Proportion of stunting, wasting and underweight in normal and elevated biomarkers.

Variable	NEO				MPO				AAT			
	Normal	Elevated	OR (95% CI)[1]	P-value[1]	Normal	Elevated	OR (95% CI)[1]	P-value[1]	Normal	Elevated	OR (95% CI)[1]	P-value[1]
Stunting												
Not stunted	73 (58.4%) [a]	189 (63.2%)			210 (60.2%)	70 (66.7%)	1.06		230 (60.5%)	47 (60.3%)	0.73	
Stunted	52 (41.6%)	110 (36.8%)	1.01 (0.64,1.58)	0.974	139 (39.8%)	35 (33.3%)	(0.65,1.77)	0.806	150 (39.5%)	31 (39.7%)	(0.42,1.26)	0.255
Wasting												
Wasted	3 (2.4%)	18 (6%)			10 (2.7%)	10 (9.5%)			17 (4.4%)	6 (7.8%)		
Not wasted	122 (97.6%)	283 (94%)	0.57 (0.13,1.85)	0.4	341 (92.3%)	95 (90.5%)	0.41 (0.15,1.14)	0.09	366 (95.6%)	71 (92.2%)	0.97 (0.35,3)	0.95
Underweight												
Underweight	15 (12%)	54 (17.9%)			51 (14.5%)	20 (18.9%)			65 (16.9%)	12 (15.5%)		
Not underweight	110 (88%)	248 (82.1%)	0.53 (0.27,0.99)	0.055	300 (85.5%)	86 (81.1%)	0.6 (0.32,1.14)	0.111	319 (83.1%)	65 (84.5%)	0.99 (0.49,2.11)	0.977

[a]Number of children (proportion)

OR=odds ratio

[1]OR (95% CI) and P-values obtained with logistic regression after adjusting for age and sex

The 3 biomarkers were combined to form a composite EED score that was used to investigate the association between EED and anthropometry measures. The composite EED score was formed as described in chapter 3.1.9. Four hundred and twenty-five fecal samples that had ELISA results for all 3 biomarkers were used to form the composite EED score. Absolute weightings from the first principal component [component with most variance (**Table 4.2.3.3)**] were used for formation of the composite EED score. The plausibility of combining MPO, NEO and AAT to make the composite EED score is visualized in **Figure 4.2.3.2** below. Highest and lowest values for all the three biomarkers clustered separately along the first principal component (**Figure 4.2.3.2 A-C**). Also, the composite EED score increased with increasing individual biomarker concentration for each of the 3 biomarkers (**Figure 4.2.3.2 D**). The scores ranged from 0 (lowest score) to 3.46 (highest score) with a mean (SD) of 1.73 (0.91). The composite EED score was categorized into low and high scores centered around the mean (1.73). Of the 425 participants with composite EED score data, 396 had corresponding HAZ score data, 398 WAZ score data and 397 WHZ score data. The Student t-test was then used to compare the distribution of HAZ, WAZ or WHZ scores between low and high composite EED scores. There were no differences in mean (SD) HAZ, WAZ and WHZ scores in children with low composite EED score compared to children with high composite EED score (**Table 4.2.3.4**). Logistic regression analysis was then used to test for associations between composite EED score and stunting, wasting or being underweight. There was no association between the composite EED score and stunting, wasting or being underweight (**Table 4.2.3.5**).

Table 4.2.3.3 Importance of the principal components (PC)

Variable	PC 1	PC 2	PC 3
Standard deviation	1.26	0.87	0.8
Proportion of variance	0.53	0.25	0.22
Cumulative proportion	0.53	0.78	1

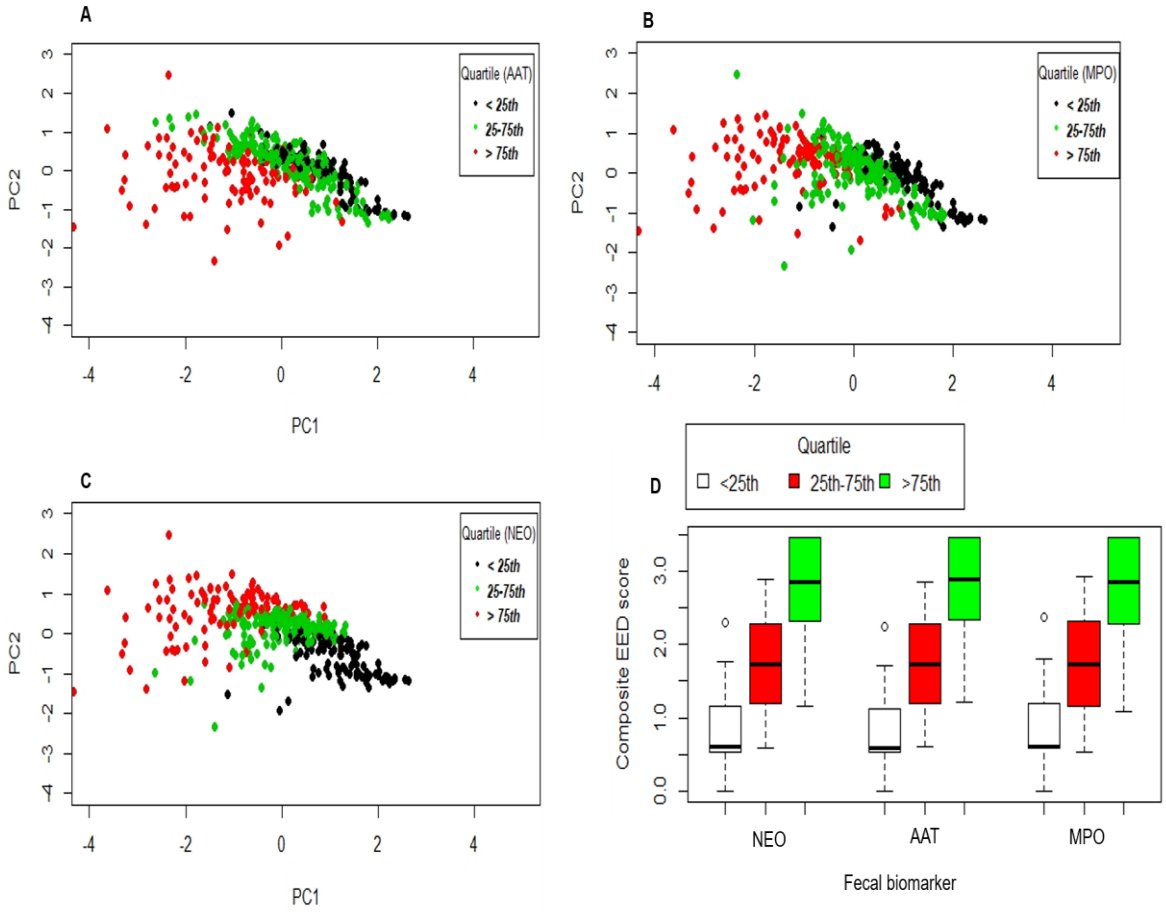

Figure 4.2.3.2 PCA plots of quartile categorized values for AAT (A), MPO (B) and NEO (C) for PC1 and PC2. (D) Boxplot of quartile categorized fecal biomarker and composite EED score.

Table 4.2.3.4 HAZ, WAZ and WHZ score distribution between low and high composite EED score.

	Composite EED score		
Variable	Low	High	P-value
Mean (SD) HAZ	-1.61 (1.49)	-1.51 (1.64)	0.22
Mean (SD) WAZ	-0.88 (1.24)	-0.96 (1.03)	0.49
Mean (SD) WHZ	-0.04 (1.24)	-0.03 (1.0)	0.97

P-values obtained with Student's t-test.

Table 4.2.3.5: Association between composite EED score and growth outcomes.

	Composite EED score		OR (95% CI)[1]	P-value[1]
Variable	Low	High		
Stunting				
Not stunted	105 (60%)	140 (63.3%)	0.89	
Stunted	70 (40%)	81 (36.7%)	(0.56,1.4)	0.61
Wasting				
Not wasted	171 (97.7%)	207 (93.2%)	0.48	
Wasted	4 (2.3%)	15 (6.8%)	(0.12,1.51)	0.22
Underweight				
Not underweight	152 (86.9%)	182 (81.6%)	0.67	
Underweight	23 (13.1 %)	41 (18.4%)	(0.38,1.15)	0.038

OR=Odds ratio.

[1]OR (95% CI) and P-values obtained with logistic regression after adjusting for age and sex.

4.3 Discussion

The present study estimated the magnitude and prevalence of intestinal inflammation and permeability in rural Malawian children using fecal biomarkers assayed by ELISA. The study also assessed associations between the fecal biomarkers of intestinal inflammation or permeability and growth in this population. The study found NEO to be elevated in almost two-thirds of the children, while MPO was raised in one-third of the children and AAT was elevated in less than one third of the children making it the least elevated biomarker in this population. No correlation was found between the biomarkers suggesting that these biomarkers measure different processes. There were differences in biomarker concentration by age; biomarker levels decreased with age. The present study also found significant differences in fecal levels of AAT and MPO but not NEO by geographic location. Fecal samples of children who resided in Namwera had higher levels of AAT and MPO compared to fecal samples of children from the other 4 geographic locations. There were no significant

differences in fecal biomarker concentration by sex. Furthermore, there was no significant association between biomarkers of EED (individually or as a composite score) and stunting or wasting, but normal NEO concentration was weakly associated with reduced chances of being underweight. These results indicate effects of age and the environment on intestinal inflammation or permeability and suggest that in this population, intestinal inflammation and permeability may not be the primary drivers of child growth inhibition.

4.3.1 The magnitude and prevalence of intestinal inflammation or permeability in rural Malawian children

Previous studies of EED have described the magnitude of intestinal inflammation and permeability indicated by fecal levels of AAT, MPO and NEO in low-income settings. More than 80% of Bangladeshi infant fecal samples were reported to have elevated concentrations of MPO, AAT and NEO relative to Western values[162,163]. In a multisite study that examined the trends in the concentration of fecal AAT, MPO and NEO in infants from low-income settings, average concentration of AAT, MPO and NEO in the infants was reported to be higher than Western reference values and the concentration of each biomarker decreased with increasing age[161]. The current study found a larger proportion of fecal samples from children to have elevated levels of NEO indicating higher levels of intestinal inflammation. MPO and AAT were elevated in one-third and one-sixth of the fecal samples respectively, and the median MPO and AAT concentration values were higher in children aged between 1 and 12 months compared to reference values. Additionally, the study found higher levels of fecal AAT, MPO and NEO in younger children. These findings are similar to data that suggests higher levels of intestinal inflammation and permeability in infants from resource limited settings[162]. Also, the inverse relationship between age and fecal levels of AAT, MPO, NEO found in this study confirms trends shown by previous studies[161].

Higher levels of fecal AAT, MPO and NEO in infants have been previously associated with breastfeeding[161]. Although the present study did not investigate associations between breastfeeding and fecal levels of AAT, MPO and NEO, the elevated biomarker levels in children aged 1 to 12 months compared to the older children may suggest a potential effect of breastfeeding on biomarker

concentration in this population as most Malawian children of this age consume breast-milk[131]. The current study did not find any relationship between the individual biomarkers, indicating that there can be high levels of one biomarker in the absence of other biomarkers. Higher levels of intestinal inflammation and permeability in children who were resident in Namwera compared to the other geographic locations suggest environmental exposures are associated with EED in this geographic location.

4.3.2 Fecal biomarkers of intestinal inflammation and permeability were not associated with growth outcomes

Intestinal inflammation and permeability are some of the key features of EED and previous studies have shown or predicted associations between high levels of MPO, AAT or NEO and growth faltering[53,54,57]. Conflicting data also exists for the relationship between these biomarkers and growth outcomes. George *et al.* did not find significant associations between higher baseline fecal levels of AAT, MPO or NEO and stunting or wasting nor did they find a relationship between baseline composite EED score and stunting[56]. Additionally, Campbell and colleagues did not find associations between composite EED score and HAZ score at 18 months of follow-up[163]. The findings by the present study are in conflict with some studies but consistent with others. Most of the studies that have reported associations between the biomarkers of intestinal inflammation or permeability and growth were longitudinal studies conducted in children less than 2 years of age and generally had larger sample sizes[53,57,161] compared to the present study and other studies that have reported conflicting data[56,163]. Nonetheless, one study that had a sample size similar to the present study reported significant associations between high fecal MPO or AAT concentration and linear growth impairment[54]. The absence of a significant association between fecal levels of AAT, MPO or NEO and growth outcomes in the present study suggests that intestinal inflammation and permeability are not predominant drivers of growth failure in this population. Additionally, the present study showed higher levels of AAT, MPO and NEO in younger children compared to older children suggesting that if associations between intestinal inflammation or permeability and child growth exist, they may be more pronounced in the early years of life as the relationship between intestinal inflammation or permeability and child growth has primarily been reported in studies conducted in infants. Also,

the inconsistencies surrounding the relationship between biomarkers of EED and child growth underscore the complexity of EED. Thus, the relationship of EED and its pathway to growth failure is likely more complex than suggested to date. Furthermore, considering that child growth is also determined by many other factors, the relationship between intestinal inflammation or permeability and child growth might be masked by other factors such as, food insecurity, prevalence of enteric infections or diarrheal diseases, social-economic status, maternal education and other determinants of growth that were not investigated in the present study.

One of the limitations for this study was the availability of fewer fecal samples that were included in the analyses compared to the total number of children enrolled by the MORDOR-morbidity baseline study, which limited generalization of results. However, this study found age-related differences in fecal biomarker concentration, which are consistent with trends reported by previous studies conducted in similar settings[161].

4.3.3 Conclusion and future work

The present study showed a higher prevalence of inflammation, indicated by fecal levels of NEO, in rural Malawian children compared to Western standards. Also, intestinal inflammation and permeability was found to be significantly higher in younger children compared to older children. These results are consistent with existing data suggesting an effect of age on intestinal inflammation and permeability. Higher levels of AAT, MPO and NEO were found in the age group corresponding to when children are breastfed and breastfeeding has previously been shown to be associated with high fecal levels of AAT and MPO[161]. Since fecal AAT in breastfeeding infants is suggested to be derived from human milk and not infant protein loss[164], future studies should assess maternal breast-milk composition in this population, investigate the impact of breast-milk constituents on fecal levels of AAT, MPO or NEO and evaluate whether the use of these biomarkers in breastfed children is ideal.

This study also found higher levels of intestinal inflammation and permeability in children resident in Namwera compared to other geographic locations. There is need to investigate if there are any environmental exposures associated with

intestinal inflammation and permeability in Namwera. To do this, studies should investigate the burden of enteric infections, WASH practices, breastfeeding patterns and exposure to contaminated foods in this population. The present study did not find an association between intestinal inflammation or permeability and growth impairment. This adds to the conflicting reports of associations between intestinal inflammation and growth. Further studies should be done in this population to assess if associations between fecal biomarkers of EED and child growth are not masked by other determinants of growth such as food insecurity and burden of enteric diseases. Furthermore, the plausibility of using the composite EED score as a disease activity score in EED needs to be validated with gold standard tests such as biopsy or endoscopy. The validation studies should also explore the mechanisms underlying the combined variability of the biomarkers that form the composite EED score.

5. Prevalence of intestinal carriage in rural Malawian children of mucolytic bacteria and bacteria associated with growth in a mouse model

5.1.1 Introduction

The human gut is colonized by bacteria that play various roles in regulating host physiological processes, one of which is nutrient utilization. Anaerobic bacteria localized in the colon ferment undigested carbohydrates thereby producing short-chain fatty acids (SCFAs) that help maintain host's energy balance[69]. Intestinal bacteria also play a role in resisting pathogen colonization. A review by Pickard *et al.* suggests that intestinal bacteria resist pathogen colonization by producing bacteriocins (bactericidal toxins) and by competing for nutrients[165]. A study investigating the effect of a probiotic bacteria-derived bacteriocin on protection against *Listeria* infection in mice showed protective effects of the bacteriocin against *Listeria monocytogenes* infection[166]. Also, *Escherichia coli* Nissle 1917, administered as a probiotic bacterium in mouse models of acute colitis, was shown to outcompete *Salmonella* Typhimurium for iron subsequently reducing its intestinal colonization[167].

Emerging evidence from murine studies indicates that the intestinal bacteria might also play a role in mediating growth. A study that assessed whether a growth phenotype could be transmitted via gut bacteria reported significant associations[75]. In this study, fecal samples were collected from Malawian twin-pairs discordant for kwashiorkor, who were less than 3 years old, had no signs of diarrhea and were not consuming antibiotics at the time of sample collection. Each sample was then transplanted into separate adult germ-free mice that had been fed a sterilized diet, typical of a rural Malawian diet a week before transplantation. After fecal transplantation, the mice were kept on the pseudo-Malawian diet for 3 weeks and then switched to ready-to-use therapeutic food (RUTF), usually given to malnourished children, for 2 weeks. The mice were switched back to the pseudo-Malawian diet after two weeks on RUTF. Mice that were transplanted with fecal samples from kwashiorkor co-twins lost more weight during the first 3 weeks of consuming a pseudo-Malawian diet compared to mice that were transplanted with fecal samples from their healthy co-twins, however, all the mice rapidly gained weight during the 2 weeks on RUTF. Re-exposure of the mice to the pseudo-Malawian diet after 2 weeks on RUTF did not show significant weight variations compared to what was shown by mice colonized with the kwashiorkor microbiota during their first exposure to the pseudo-Malawian

diet. Comparison of the fecal microbiota composition between the two groups of mice during the first 3 weeks on the pseudo-Malawian diet showed no significant differences in the abundance of genera *Bifidobacterium* and *Ruminococcus*. There was prominent increase however in the abundance of genera *Bifidobacterium* (*B. longum* and *B. bifidum*), *Ruminococcus* (*R. torques*) and *Faecalibacterium* (*F. prausnitzii*), during the 2 weeks on RUTF, in mice given fecal microbiota from malnourished children compared to recipients of fecal samples from healthy co-twins. These results indicate an association between a discordant weight loss phenotype and a combination of a Malawian diet and a kwashiorkor microbiota. Another corresponding study, conducted to test the association between gut microbiota development and undernutrition, reported a relationship between gut microbiota and growth phenotypes in a mouse model[76]. In this study, fecal samples from rural Malawian children aged 6 or 18 months, representing either a healthy or undernourished growth phenotype, were transplanted into germ-free mice and followed up for 5 weeks. The mice were put on a sterile, typical rural Malawian diet, 3 days before fecal transplantation and remained on that diet throughout the study. Mice that harbored microbiota from healthy Malawian children gained more total body weight and lean mass compared to mice colonized with microbiota from undernourished children for both the 6-month and 18-month age bins. There were no significant associations between bacteria diversity and growth phenotypes reported. However, the study identified specific bacteria associated with weight gain in mice, which included *Bifidobacterium longum, Faecalibacterium prausnitzii* and *Dorea formicigenerans*.

A few studies, conducted in the human population, have also reported associations between the gut microbiota and growth phenotypes. A study that characterized the fecal microbiota of malnourished and healthy Bangladeshi children reported a persistent reduction of intestinal bacterial diversity in malnourished children compared to healthy children[168]. Additionally, the study reported a lower relative abundance of *Bifidobacterium* in malnourished children compared to healthy children. These results suggest a potential role of intestinal bacteria on a host's nutritional status, which can subsequently affect growth and warrant further research to explore associations between gut bacteria and growth in the human population.

There is also emerging evidence linking a recently discovered gram-negative mucin-degrading bacterium, *Akkermensia muciniphila*, to gut health and body weight. One *in vitro* study that used human colonic cell-lines to investigate the interaction of *A. muciniphila* with the host mucosa showed that *A. muciniphila* adhered to the intestinal epithelium and reinforced enterocyte integrity, indicating the capability of this bacterium to strengthen intestinal barrier[169]. Another study also reported an association between low abundance of *A. muciniphila* and Crohn's disease (CD) or ulcerative colitis (UC) in *in vitro* studies of inflammatory bowel disease[170]. There is also evidence from murine and human studies indicating an association between intestinal carriage of *A. muciniphila* and body weight[171-173]. A study that was conducted in mice to understand the physiological roles of *A. muciniphila* in obesity reported an inverse relationship between intestinal carriage of *A. muciniphila* and body weight[174]. Similarly, another study in Swedish pre-school children reported significantly lower abundance of *A. muciniphila* in overweight or obese children[175]. Nevertheless, the role of *A. muciniphila* in gut health and growth of children living in low-income settings, who are at risk of developing EED, remains to be studied.

Drawing from the above literature, this study utilized fecal samples from rural Malawian children to describe the prevalence of the following bacteria that have been associated with gut health and weight gain and had commercial PCR kits available: *Akkermensia muciniphila, Bifidobacterium longum, Dorea formicigenerans,* and *Faecalibacterium prausnitzii*. The study also explored associations between intestinal carriage of these bacteria, EED and growth outcomes.

5.1.2 Study design and analyses

The current study utilized data and biological samples collected by the baseline survey of the MORDOR-Malawi morbidity study, the details of which are described in chapter 3. Briefly, the baseline survey sought to recruit 1200 randomly selected children aged between 1-59 months and residing in 5 geographical zones of Mangochi district, Malawi. One-thousand-ninety children from a randomization list of 1704 names were enrolled into the MORDOR-Malawi morbidity study at baseline. Anthropometry measurements (height and weight),

demographics (age and sex) and fecal samples were collected as described in chapter 3. Height and weight measurements and demographics were subsequently used to calculate HAZ, WHZ and WAZ scores using WHO growth standards[34].

Out of the 1090 enrolled children, 709 provided fecal samples for the study of antimicrobial resistance in MORDOR-Malawi morbidity, of which 612 were available for inclusion in the current study. Total, genomic DNA was extracted from the fecal samples as described in chapter 3.1.8.4. The extracted genomic DNA was amplified by qPCR (detailed in chapter 3.1.8.5) to describe fecal carriage of the following bacteria: *Akkermensia muciniphila, Bifidobacterium longum, Dorea formicigenerans,* and *Faecalibacterium prausnitzii*. The qPCR results were classified as positive or negative using a recently published method[147], with modifications as described in chapter 3.1.9. Additionally, 16S rRNA PCR described in chapter 3.1.8.6 was performed. To determine relative bacterial abundance, amplification data for each of the 4 bacteria was normalized relative to the bacterial 16S rRNA gene in the same sample using the delta C_T method[153]. Thus, the resultant delta C_T values for each bacterium were considered as the relative abundance of that bacterium in the fecal sample.

To describe the prevalence of carriage of defined bacteria, proportions of fecal samples with a positive qPCR result for each of the defined bacteria were calculated. The fecal samples were then stratified by age, sex, geographic location, growth outcome (stunting, wasting and underweight) and proportions of bacterial carriage were calculated in each group. Stunting, wasting and underweight were defined as described in section 3.1.9 of chapter 3. Fisher's Exact or Pearson's chi-square tests were used to explore associations between bacterial carriage and sex, age, geographic location, growth outcomes and EED. The associations between bacterial carriage and biomarkers of EED or growth outcomes were also explored using regression analyses adjusting for age and sex. The delta C_T values for each bacterium were used as a predictor in regression analyses to explore associations between relative bacterial abundance and growth outcomes or biomarkers of EED.

5.2 Results

5.2.1 Baseline characteristics of participants

The MORDOR-Malawi morbidity study enrolled 1090 children at baseline however only 709 of these participated in the fecal sampling. Of the 709 samples collected, 612 fecal samples were available for inclusion in this study (**Figure 3.1.3.2** of chapter 3). Characteristics of all children enrolled in MORDOR-morbidity and those included in the final analyses of this study are shown in Table **5.2.1.1** below. Participant sex, age, height, weight, HAZ, WHZ and WAZ scores, and proportion of children residing in the Makanjira, Mangochi and Monkey Bay zones were comparable between all enrolled participants and those included in this study, however, a larger proportion of children included in this analysis resided in Chilipa zone and a smaller proportion in Namwera zone compared to all enrolled participants.

Table 5.2.1.1 Characteristics of participants who were included in analyses compared to all enrolled participants.

Variable	All enrolled participants	Participants with fecal sample included in the present analyses	P-value
Number of participants	1090	612	
Male sex N (%)	526 (48.26%)	299 (48.78%)	0.85¥
Height, cm	83.36 (12.43)	84.49 (12.06)	0.07*
Weight, kg	11.34 (3.13)	11.6 (3.06)	0.09*
Age, months	29.59 (16.42)	31.1 (16.36)	0.07*
HAZ score	-1.48 (1.81)	-1.49 (1.81)	0.9*
WHZ score	0.11 (3.51)	0.18 (4.36)	0.74*
WAZ score	-0.86 (1.28)	-0.87 (1.25)	0.88*
Geographical location			
Chilipa N (%)	235 (21.56%)	168 (27.41%)	0.005¥
Makanjira N (%)	225 (20.64%)	131 (21.37%)	0.45¥
Mangochi N (%)	237 (21.74%)	106 (17.29%)	0.09¥

Monkey Bay N (%)	192 (17.61%)	133 (21.7%)	0.12
Namwera N (%)	201 (18.44%)	75 (12.23%)	0.0001

Data are presented as mean (SD) unless otherwise stated.

**Denotes p-values obtained from Student's t-test while ¥denotes p-values obtained from sample proportion test.*

5.2.2 Prevalence of bacterial carriage in the fecal samples

Proportions of fecal samples with a positive qPCR result for each of the 4 bacteria were calculated (**Table 5.2.2.1**). *D. formicigenerans and F. prausnitzii* were the most prevalent bacteria; *D. formicigenerans* was present in 79% of the samples while *F. prausnitzii* was present in 98% of the samples. *B. longum* was present in just over one third of the fecal samples making it the least prevalent bacterium tested. Fecal samples were stratified by age, sex and geographic location and bacterial carriage was determined in each category. There was no difference in the prevalence of *F. prausnitzii*, *B. longum* and *A. muciniphila* between male and female children, however, a higher proportion of fecal samples from male children were positive for *D. formicigenerans* compared to the female children (**Table 5.2.2.2**). Prevalence of *D. formicigenerans, F. prausnitzii* and *B. longum* but not *A. muciniphila* were comparable by geographic location (**Table 5.2.2.3**). A larger proportion (56%) of fecal samples from children residing in Chilipa zone were positive for *A. muciniphila* compared to fecal samples from other zones while a lower proportion (26%) of fecal samples from children in Mangochi zone were positive for this bacterium. Child age, in months, was categorized into 5 groups (1-12, 13-24, 25-36, 37-48 and 49-60) to compare bacterial carriage. Fecal bacterial carriage for all the 4 bacteria was different by age (**Table 5.2.2.4**). *B. longum* was more prevalent in fecal samples from the younger children while *D. formicigenerans, F. prausnitzii* and *A. muciniphila* were more prevalent in the older children.

Table 5.2.2.1 Prevalence of bacterial carriage in the fecal samples

Variable	Proportion of positive samples (n)
B. longum	29.2 % (179)
A. muciniphila	43.1 % (264)
D. formicigenerans	78.8 % (483)
F. prausnitzii	98.4 % (603)

Total number of fecal samples=612.

Table 5.2.2.2 Bacterial carriage by sex

Variable	Sex		P-value[1]
	Female (n=314)	Male (n=299)	
B. longum positive N (%)	96 (30.57)	83 (27.76)	0.5
A. muciniphila positive N (%)	132 (42.04)	132 (44.15)	0.65
D. formicigenerans positive N (%)	235 (74.84)	248 (82.94)	0.019
F. prausnitzii positive N (%)	307 (97.77)	296 (99)	0.34*

*[1]All P-values were obtained from Pearson's chi-square test except *denoted P value that was obtained from Fisher's Exact test.*

Table 5.2.2.3 Bacterial carriage by geographic location

Variable	Geographical location					P-value[1]
	Chilipa (n=168)	Makanjira (n=131)	Mangochi (n=106)	Monkey Bay (n=133)	Namwera (n=75)	
B. longum positive N (%)	52 (30.95)	35 (26.72)	28 (26.42)	40 (30.08)	24 (32)	0.85
A. muciniphila positive N (%)	93 (55.36)	53 (40.86)	28 (26.42)	56 (42.11)	34 (45.33)	0.0001
D. formicigenerans positive N (%)	131 (77.98)	110 (83.97)	80 (75.47)	110 (82.71)	52 (69.33)	0.08
F. prausnitzii positive N (%)	164 (97.62)	128 (97.71)	106 (100)	131 (98.5)	74 (98.67)	0.59*

[1]All P-values were obtained from Pearson's chi-square test except *denoted P value that was obtained from Fisher's Exact test.

Table 5.2.2.4 Bacterial carriage by age

Variable	Age in months					P-value[1]
	1-12 (n=104)	13-24 (n=134)	25-36 (n=120)	37-48 (n=144)	49-60 (n=111)	
B. longum positive N (%)	85 (81.73)	61 (45.52)	17 (16.5)	12 (8.33)	4 (3.6)	2.2e-16
A. muciniphila positive N (%)	27 (25.96)	64 (47.76)	63 (52.5)	60 (41.67)	50 (45.05)	0.001
D. formicigenerans positive N (%)	44 (42.31)	109 (81.34)	105 (87.5)	122 (84.72)	103 (92.79)	2.2e-16
F. prausnitzii positive N (%)	99 (95.19)	132 (98.51)	120 (100)	143 (99.31)	109 (98.2)	0.06*

[1]All P-values were obtained from Pearson's chi-square test except *denoted P value that was obtained from Fisher's Exact test.

5.2.3 *Intestinal carriage of B. longum and D. formicigenerans was associated with biomarkers of EED*

Analyses were conducted to test for associations between bacterial carriage and biomarkers of EED. Individual biomarkers (AAT, NEO and MPO) and the composite EED score described in chapter 4 were used in this analysis. Briefly, out of the 612 participants who were included in the analysis of prevalence of bacterial carriage, 478 had corresponding ELISA results for at least one of the 3 fecal biomarkers and so were included in the analysis of the associations between bacterial carriage and individual biomarkers of EED. As presented in the analyses of chapter 4, 71% (295/417) of fecal samples had elevated NEO concentration while 23% (104/449) and 16% (74/454) had elevated concentrations for MPO and AAT respectively. Logistic regression analysis was conducted to explore associations between bacterial carriage and biomarker concentration (categorized as normal or elevated as described in chapter 4) adjusting for age, location and sex. In the logistic regression models, bacterial carriage was used as a predictor variable while biomarker concentration was used as a binary response variable. The analysis found no associations between fecal carriage of *A. muciniphila* or *F. prausnitzii* and any of the 3 biomarkers of EED. However, there was evidence of an association between fecal carriage of *B. longum* and all the 3 biomarkers. Being positive for *B. longum* carriage increased the odds of having elevated biomarker concentrations (vs normal concentrations) by factors of 2.6, 2.6 and 4.7 for MPO, AAT and NEO respectively. Also, fecal carriage of *D. formicigenerans* was associated with normal MPO concentration; fecal samples that were positive for *D. formicigenerans* had 0.3 odds of having elevated MPO concentration compared to those that were negative for *D. formicigenerans* (**Table 5.2.3.1**).

In chapter 4, fecal biomarker concentration was shown to be associated with age. In this chapter, age was again associated with bacterial carriage. To mitigate for confounding, age in months was stratified into 4 groups (0-12, 12-24, 24-36 and 36-60) and associations between bacterial carriage and the biomarkers were explored in each age group. *B. longum* carriage in the younger age groups (0-12 and 12-24) was associated fecal concentrations of MPO and NEO but not in the older age groups (> 24 months) [**Table 5.2.3.2**]. In the 0-12 age group, *B. longum* carriage increased the odds of having elevated MPO concentration (vs normal

concentration) by a factor of 7.4 while being positive for *B. longum* in the 12-24 age group increased the odds of having elevated NEO concentration (vs normal concentration) by a factor of 17.5. *D. formicigenerans* carriage was associated with MPO concentration in the youngest age group after adjusting for sex and geographic location (0-12 months, OR=0.18, 95% CI [0.04,0.59], P=0.007); fecal samples that were positive for *D. formicigenerans* in the 0-12 age group had 0.18 odds of having elevated MPO concentration compared to those that were negative for *D. formicigenerans*. There were no significant associations between *D. formicigenerans* carriage and MPO concentration in the other 3 age groups (12-24 months, OR=0.8, 95% CI [0.2,3.3], P=0.7, 24-36 months, OR=1.1, 95% CI [0.1,23.2], P=0.96 and 36-60 months, OR=0.3, 95% CI [0.1,1.03], P=0.044).

Table 5.2.3.1 Association between *B. longum* carriage and biomarkers of EED

	MPO		AAT		NEO		MPO		AAT		NEO	
Variable	Normal (n=345)	Elevated (n=104)	Normal (n=380)	Elevated (n=74)	Normal (n=122)	Elevated (n=295)	OR[a] (95% CI)	P-value[a]	OR[a] (95% CI)	P-value[a]	OR[a] (95% CI)	P-value[a]
B. longum n (%)	60 (17.4)	59 (56.7)	72 (18.9)	47 (63.5)	7 (5.7)	98 (33.2)	2.6 (1.4,4.6)	0.002	2.6 (1.4,5.1)	0.004	4.7 (2.1,12.3)	0.0004
A. muciniphila n (%)	159 (46.1)	41 (39.4)	176 (46.3)	27 (36.5)	61 (50)	133 (45.1)	0.9 (0.5,1.5)	0.619	0.8 (0.4,1.3)	0.354	0.8 (0.5,1.3)	0.407
D. formicigenerans n (%)	307 (89)	66 (63.5)	328 (86.3)	48 (64.9)	112 (91.8)	244 (82.7)	0.3 (0.2,0.6)	0.001	0.7 (0.4,1.4)	0.298	0.7 (0.3,1.5)	0.427
F. prausinitzii n (%)	340 (98.6)	102 (98.1)	374 (98.4)	73 (98.7)	121 (99.2)	291 (98.6)	0.9 (0.1,7.6)	0.89	2.5 (0.3,53.6)	0.454	0.5 (0.03,4)	0.585

CI= confidence interval, OR=odds ratio.

n (%) = Number of fecal samples (proportion) positive.

[a]Odds ratio (95% CI) and P-values were obtained with logistic regression after adjusting for age, location and sex. Using the Bonferroni correction for multiple testing, associations with a P-value less than 0.0125 were considered statistically significant.

Table 5.2.3.2 Association between bacterial carriage and biomarkers of EED stratified by age

Age (months)	MPO		AAT		NEO		MPO		AAT		NEO	
	Normal	Elevated	Normal	Elevated	Normal	Elevated	OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value
	¥n=23	n=44	n=34	n=31	n=4	n=50						
0 – 12 *n (%)	13 (56)	40 (91)	23 (68)	27 (87)	3 (75)	40 (80)	7.4 (1.9,34.5)	**0.006**	3.02 (0.81,13.1)	0.112	1.4 (0.02,14)	0.804
	n=72	n=30	n=79	n=24	n=17	n=80						
12 – 24 n (%)	29 (40)	16 (53)	30 (38)	16 (67)	1 (6)	41 (51)	1.7 (0.7,4.3)	0.268	3.1 (1.2,8.6)	0.023	17.5 (3.26,324.8)	**0.007**
	n=73	n=10	n=79	n=8	n=28	n=53						
24 – 36 n (%)	7 (10)	2 (20)	6 (8)	3 (38)	0	7 (13)	2.1 (0.3,11.8)	0.427	11.9 (1.6,121)	0.019	2.8 (0.03,14.2)	0.991
	n=177	n=20	n=188	n=11	n=73	n=112						
36 – 60 n (%)	11 (6)	1 (5)	13 (7)	1 (9)	3 (4)	10 (9)	0.68 (0.04,11.8)	0.727	1.28 (0.06,8.5)	0.822	2.4 (0.7,11.3)	0.204

**n (%) = Number (proportion) positive for B. longum*

¥Number with normal or elevated biomarker concentration

CI= confidence interval, OR=odds ratio. [a]Odds ratio (95% CI) and P-values were obtained with logistic regression after adjusting for location and sex. Using the Bonferroni correction for multiple testing, associations with a P-value less than 0.0125 (highlighted in bold) were considered statistically significant.

Of the 478 participants who had ELISA results, only 389 had ELISA results for all the 3 biomarkers from which the composite EED score could be calculated. Thus, data from these 389 participants was used to explore the associations between bacterial carriage and the composite EED score. Composite EED scores were categorized into low and high scores centered around the mean score (1.73). Logistic regression analysis, adjusted for age and sex, was conducted to explore associations between bacterial carriage and composite EED score; bacterial carriage was used as a predictor variable while the composite EED score was used as a binary response variable in the logistic regression model. The analysis showed no associations between fecal carriage of *A. muciniphila*, *F. prausnitzii* or *D. formicigenerans* and the composite EED score, however, fecal samples that were positive for *B. longum* had 7.0 increased odds of having high composite EED scores relative to those that were negative for *B. longum* (**Table 5.2.3.3**).

Table 5.2.3.3 Association between bacterial carriage and composite EED score

Variable	Composite EED score			
	Low (n=175)	High (n=214)	OR (95% CI)[1]	P-value[1]
B. longum	9 (5.1)	90 (42.1)	7.0 (3.3,16.3)	1.08e-06
A. muciniphila	83 (47.4)	96 (44.9)	1.0 (0.6,1.6)	0.979
D. formicigenerans	162 (92.6)	169 (79)	0.7 (0.3,1.5)	0.06
F. prausnitzii	172 (98.3)	212 (99.1)	1.4 (0.2,12.6)	0.73

CI= confidence interval.

OR=Odds ratio.

[1]Odds ratio (95% CI) and P-values obtained with logistic regression after adjusting for age, location and sex.

5.2.4 Intestinal carriage of F. prausnitzii and B. longum was associated with growth outcomes

Associations between bacterial carriage and growth outcomes were explored using regression analyses. HAZ, WHZ and WAZ scores were used as continuous

response variables in linear regression analyses to explore associations between bacterial carriage and Z scores. Linear regression analysis, adjusted for age and sex, showed no associations between any of the 4 bacteria and HAZ or WHZ scores, however, *B. longum* and *F. prausnitzii* were associated with lower WAZ (**Table 5.2.4.1**). The Z scores were then categorized into stunted or not-stunted, wasted or not-wasted and underweight or not-underweight as described in chapter 3.1.9. Comparison of bacterial carriage by growth outcome showed similar proportions of *D. formicigenerans, F. prausnitzii* and *A. muciniphila* between fecal samples from stunted and non-stunted, wasted and not wasted and underweight or not-underweight children (**Table 5.2.4.2**). Logistic regression analysis, adjusting for age and sex, showed no association between intestinal carriage of *D. formicigenerans, F. prausnitzii*, *A. muciniphila* and stunting, wasting or being underweight, however, *B. longum* carriage was associated with 2.7 increased odds of being underweight compared to not being underweight (**Table 5.2.4.2**).

Table 5.2.4.1 Association between bacterial carriage and Z-scores

Variable	HAZ		WHZ		WAZ	
	Coefficient (95% CI) [a]	**P-value[a]**	**Coefficient (95% CI) [a]**	**P-value[a]**	**Coefficient (95% CI) [a]**	**P-value[a]**
B. longum	-0.26 (-0.0.64,0.12)	0.175	0.49 (-0.45,1.44)	0.31	-0.49 (-0.75, -0.23)	**0.0003**
A. muciniphila	- 0.24 (-0.53,0.03)	0.083	-0.35 (-1.05,0.35)	0.33	-0.14 (-0.34, 0.05)	0.15
D. formicigenerans	- 0.08 (-0.44,0.28)	0.656	-1.07 (-1.97, -0.17)	0.02	-0.06 (-0.31,0.2)	0.66
F. prausnitzii	-0 .01 (-1.1, 1.08)	0.985	-3.04 (-5.78, -0.31)	0.029	-1.53 (-2.23, -0.77)	**9e-05**

CI= confidence interval.

[a]Coefficients (95% CI) and P-values obtained from a linear regression analysis after adjusting for age and sex. Using the Bonferroni correction for multiple testing, associations with a P-value less than 0.0125 (highlighted in bold) were considered statistically significant.

Table 5.2.4.2 Association between bacterial carriage and growth outcomes

Variable	Stunting		Wasting		Underweight		Stunting		Wasting		Underweight	
	Stunted (n=229)	Not stunted (n=384)	Wasted (n=28)	Not wasted (n=585)	Yes (n=93)	No (n=520)	OR[a] (95% CI)	P-value[a]	OR[a] (95% CI)	P-value[a]	OR[a] (95% CI)	P-Value[a]
B. longum n (%)	55 (24%)*	124 (32.3%)	16 (57.1%)	163 (28.9%)	35 (37.6%)	144 (27.7%)	0.99 (0.62,1.57)	0.97	1.66 (0.63,4.53)	0.31	2.7 (1.5,5.1)	0.001
A. muciniphila n (%)	107 (46.8%)	157 (40.9%)	10 (35.7%)	254 (43.4%)	47 (50.5%)	217 (41.7%)	1.21 (0.86,1.69)	0.27	0.87 (0.37,1.93)	0.73	0.87 (0.37,1.93)	0.15
D. formicigenerans n (%)	190 (83%)	293 (76.3%)	18 (64.3%)	465 (79.5%)	74 (79.6%)	409 (78.6%)	1.18 (0.76,1.85)	0.47	0.83 (0.34,2.09)	0.67	0.83 (0.34,2.09)	0.87
F. prausinitzii n (%)	226 (99%)	377 (98.2%)	28 (100%)	575 (98.3%)	93 (100%)	510 (98.1%)	1.13 (0.3,5.38)	0.86	3.4 (0.006,6.77)	0.99	3.1 (0.006,6.8)	0.98

**Number of positive samples (proportion of positive samples).*

[a]P-value obtained with logistic regression analysis adjusted for age and sex.

Using the Bonferroni correction for multiple testing, associations with a P-value less than 0.0125 were considered statistically significant.

5.2.5 *Relative bacterial abundance was associated with biomarkers of EED but not growth outcomes*

Relative bacterial abundance was determined by normalizing amplification data for the specific bacteria relative to amplification of total bacterial DNA in each sample. Only samples that had a target from the species-specific PCR (detailed in chapter 3.1.8.5) amplified before 40 cycles were used to calculate relative bacterial abundance. Six hundred and twelve samples had *D. formicigenerans* or *F. prausnitzii* amplified before 40 cycles, 603 *B. longum* and 352 *A. muciniphila* and therefore were included in this analysis. Logistic regression analysis was conducted to explore associations between relative bacterial abundance and biomarkers of EED, stunting, wasting and underweight while linear regression analysis was conducted to explore the relationship between relative bacterial abundance and Z scores. Both regression models were adjusted for age, sex or geographic location. Exploratory analyses on the relationship between relative bacterial abundance and biomarkers of EED showed no relationship between relative abundance of *D. formicigenerans* or *A. muciniphila* and all the 3 biomarkers of EED. However, there was a significant association between relative abundance of *B. longum* and MPO or NEO (**Table 5.2.5.1**). An increase in relative abundance of *B. longum* was associated with increased odds of having elevated concentration of MPO (odds ratio of 1.1, *P*=0.0015) and NEO (odds ratio of 1.1, *P*=0.017). Additionally, relative abundance of *F. prausnitzii* and fecal concentrations of MPO and AAT were associated. An increase in relative abundance of *F. prausnitzii* was associated with reduced odds of having elevated MPO (odds ratio of 0.9, P= 0.007) and AAT (odds ratio of 0.89, *P*=0.01). There was no association between relative abundance of *A. muciniphila, F. prausnitzii, D. formicigenerans* and the composite EED score, but, relative abundance of *B. longum* and composite EED score were associated (**Table 5.2.5.2**). An increase in relative abundance of *B. longum* was associated with increased odds of high composite EED score (odds ratio=1.13, *P*=0.0004). There was no relationship between relative bacteria abundance and HAZ or WAZ scores nor was there an association between relative bacteria abundance and stunting, wasting or being underweight after adjusting for age and sex. *B. longum* and *F. prausinitzii,* however, were associated with WHZ score (**Table 5.2.5.3**). A unit increase in the relative abundance of *B. longum* was associated with a 0.12 increase in WHZ

score whereas increase in the relative abundance of *F. prausnitzii* was associated with a 0.11 decrease in WHZ score.

Table 5.2.5.1 Association between relative bacterial abundance and biomarkers of EED

	MPO		AAT		NEO	
Variable	OR (95% CI)	P-value[1]	OR (95% CI)	P-value [1]	OR (95% CI)	P-value [1]
B. longum	1.1 (1.0, 1.2)	0.0015	1.1 (1,1.1)	0.073	1.1 (1.02,1.2)	0.017
A. muciniphila	1.0 (0.9, 1.1)	0.868	1.1 (1.0,1.2)	0.114	1.0 (0.9,1.1)	0.298
D. formicigenerans	1.0 (0.94, 1.14)	0.496	1.1 (0.96,1.15)	0.274	0.99 (0.9,1.1)	0.779
F. prausnitzii	0.9 (0.82, 0.97)	0.007	0.9 (0.82,0.97)	0.01	0.94 (0.87,1.02)	0.137

CI= confidence interval, OR=odds ratio.

[1]OR (95%CI) and P-values obtained from logistic regression after adjusting for age, location and sex.

Table 5.2.5.2 Association between relative bacterial abundance and composite EED score

	Composite EED score	
Variable	OR (95% CI)[1]	P-value[1]
B. longum	1.13 (1.06,1.22)	0.0004
A. muciniphila	1.07 (0.99,1.17)	0.09
D. formicigenerans	0.99 (0.9, 1.04)	0.754
F. prausnitzii	0.9 (0.8,0.98)	0.013

CI= confidence interval, OR=Odds ratio.

[1]Odds ratio (95% CI) and P-values obtained with logistic regression after adjusting for age, location and sex.

Table 5.2.5.3 Association between relative bacterial abundance and Z-scores

	HAZ		**WHZ**		**WAZ**	
Variable	Coefficient (95% CI)[1]	P-value[1]	Coefficient (95% CI)[1]	P-value[1]	Coefficient (95% CI)[1]	P-value[1]
B. longum	0.001 (-0.03,0.04)	0.961	0.12 (0.02,0.23)	0.024	-0.01 (-0.04,0.01)	0.391
A. muciniphila	-0.02 (-0.06,0.03)	0.476	0.01 (-0.03,0.05)	0.709	-0.004 (-0.037,0.029)	0.808
D. formicigenerans	0.01 (-0.03,0.06)	0.579	0.02 (-0.01,0.05)	0.204	0.02 (-0.01,0.048)	0.21
F. prausinitzii	0.004 (-0.04,0.037)	0.845	-0.11 (-0.22,-0.01)	0.031	0.02 (-0.01,0.04)	0.301

CI= confidence interval.

[1]Denotes regression coefficient and P-value obtained with linear regression after adjusting for age and sex.

5.2.6 *The effect of D. formicigenerans carriage in EED was dependent on B. longum carriage*

In the above results, *B. longum* carriage and relative abundance of *B. longum* were both significantly associated with biomarkers of EED (individually or as a composite score). *B. longum* carriage was also associated with WAZ while relative abundance of *B. longum* was associated with WHZ. Additionally, intestinal carriage of *B. longum* or *D. formicigenerans* was associated with MPO concentration. *B. longum* carriage or relative abundance and composite EED score were then included as exposures in a multi-variable linear regression analysis, together with age and sex, to test the link between *B. longum* carriage or relative abundance, EED and WAZ or WHZ. There was no relationship between *B. longum* carriage, EED and WAZ neither was there a relationship between relative abundance of *B. longum*, EED and WHZ. In another multi-variable logistic regression model, *B. longum* and *D. formicigenerans* were also included as exposures, with age and sex, to test the independence of the associations between intestinal carriage of *B. longum* or *D. formicigenerans* and EED. Intestinal carriage of *B. longum*, but not *D. formicigenerans*, was associated with 6.33 increased odds of having high composite EED scores compared to low composite EED scores (**Table 5.2.6.1**).

Table 5.2.6.1 Multi-variable logistic regression models for composite EED score in the presence of *B. longum*, *D. formicigenerans*, age and male sex

	Composite EED score	
Variable	OR (95% CI)	P-value
B. longum	6.33 (3.02, 14.59)	3.4e-06
D. formicigenerans	0.58 (0.27,1.24)	0.169
Age	0.96 (0.94,0.98)	1.1e-5
Sex (Male)	0.92 (0.58,1.46)	0.725

OR = odds ratio

CI= confidence interval

5.3 Discussion

The present study described the prevalence of intestinal carriage *Akkermensia muciniphila, Bifidobacterium longum, Dorea formicigenerans,* and *Faecalibacterium prausnitzii* in rural Malawian children. The associations between intestinal carriage of these bacteria and biomarkers of EED or child growth were also explored. *F. prausnitzii* and *D. formicigenerans* were found to be the most prevalent of the bacteria studied, with *F. prausnitzii* being present in 98% of all fecal samples. *D. formicigenerans* was present in over two-thirds of the fecal samples tested while *A. muciniphila* was present in almost half of the children and *B. longum* was the least prevalent of bacteria tested; just over one-third of the children carried this bacterium. Comparison of bacterial carriage by age showed significant differences. *B. longum* was more prevalent in younger children compared to older children while *D. formicigenerans* was more prevalent in older children. Additionally, prevalence of *D. formicigenerans* was higher in fecal samples from male children compared to female children. Fecal samples of children residing in Mangochi had a significantly lower proportion of *A. muciniphila* compared to children from the other 4 geographic locations. Intestinal carriage and relative abundance of *B. longum* were associated with elevated individual biomarker concentration and high composite EED scores while carriage of *D. formicigenerans* was associated with normal MPO concentration and tended to be associated with reduced chances of having high composite EED scores. The study also found an inverse relationship between relative abundance of *F. prausnitzii* and AAT or MPO concentrations. Analyses of the relationship between intestinal carriage of *B. longum, F. prausnitzii* and weight metrics showed associations. Intestinal carriage of *B. longum* and *F. prausnitzii* was associated with lower WAZ. Additionally, increased relative abundance of *B. longum* correlated with an increase in WHZ while increased relative abundance of *F. prausnitzii* was associated with lower WHZ. Multi-variable regression analysis showed no relationship between intestinal carriage or relative abundance of *B. longum*, EED and WAZ or WHZ, however, *B. longum* carriage was shown to negate the association between *D. formicigenerans* carriage and biomarkers of EED. These findings suggest a potential role of individual bacteria species in EED development and as mediators of weight-related growth in rural

Malawian children. Additionally, the results suggest that the effects of *D. formicigenerans* in EED are not independent.

5.3.1 Prevalence of bacterial carriage in children

F. prausnitzii is one of the most common and abundant intestinal bacteria in the human gut[176]. This bacterium has been shown to be present in very small proportions in infants during the first 6 months of life, with a gradual increase in abundance at 7 months of life that doubled during the second year of life[177]. Studies that used a machine-learning-based approach to define a healthy gut microbiota of Bangladeshi or Malawian children identified *F. prausnitzii* as one of the most important age discriminatory bacteria and confirmed its low abundance in early life and subsequent increase[76,168]. Similarly, *D. formicigenerans* was shown to be higher in abundance after 10 months of age in Bangladeshi children[168]. The present study investigated the prevalence of bacterial carriage in children less than 5 years using qPCR. The study found a larger proportion of fecal samples that were positive for *F. prausnitzii* and *D. formicigenerans*. Prevalence of *D. formicigenerans* increased with age while *F. prausnitzii* prevalence showed a trend that increased with age. These results reflect the age prevalence and abundance reported by other studies that have used real-time PCR and 16S rRNA gene sequencing [76,168,177]. This study also found that *D. formicigenerans* tended to be more prevalent in male children compared to female children. To date, there is no study that has reported a relationship between *D. formicigenerans* carriage and sex. Since there is limited data available on the associations between intestinal bacteria and sex in children, further large-scale studies are needed to validate the effect of sex on *D. formicigenerans* carriage in children and indeed its significance.

This study also detected *A. muciniphila* in almost half of the fecal samples tested. *A. muciniphila* is a recently discovered gram-negative bacterium that produces several enzymes that degrade mucin (mucous lining of the gut) subsequently providing carbon and nitrogen to other intestinal bacteria[178]. Collado *et al.* reported that *A. muciniphila* colonized the intestinal tract early in life and developed close to the level observed in healthy adults within 1 year[179]. The finding from the present study that *A. muciniphila* was present in one-third of

children aged between 1 and 12 months supports the data indicating early gut colonization by *A. muciniphila*. Additionally, the observation that the proportion of fecal samples positive for *A. muciniphila* doubled in age groups older than 12 months is in agreement with the finding that indicated increased abundance of *A. muciniphila*, close to the healthy adult level, within 1 year of life[74]. The current study also found significant differences in *A. muciniphila* carriage by geographic location. The proportion of *A. muciniphila* carriage was significantly lower in children from Mangochi compared to the other 4 locations (Chilipa, Makanjira, Monkey and Namwera) where bacterial carriage was comparable. Mangochi can be considered a peri-urban setting in comparison to the other 4 rural geographical zones so this finding suggests an environmental effect on *A. muciniphila* carriage.

B. longum is considered a beneficial bacteria that is widely used in developing probiotics[180]. Khonsari *et al.* reported higher abundance of *B. longum* in infants compared to adults[181] suggesting that *B. longum* colonizes the infant gut more than that of older children. The current study found a lower prevalence of intestinal carriage of *B. longum* than other studies. One explanation for this could lie in the number of infant participants as the current study had a larger proportion of older children. The observation that *B. longum* carriage was more often found in children younger than 12 months compared to the older age groups supports data from previous studies that indicated higher numbers of *B. longum* in infants compared to older children[64,68,181]. The higher abundance of *B. longum* in children aged 1-12 months may be attributed to breastfeeding, which is common in this age group. *B. longum* has been shown to be present in breast-milk[182,183].

5.3.2 B. longum and D. formicigenerans were associated with biomarkers of EED

There is limited data on the associations between intestinal bacteria and biomarkers of EED. A study that was conducted to identify the gut bacterial community structure associated with moderate to severe EED (defined by an L:M ratio test) in Malawian children did not document any relationship between *B. longum* or *D. formicigenerans* and L:M ratios[59]. Although there is no data available indicating the role of *B. longum* or *D. formicigenerans* in EED, studies of IBD have reported mixed results on the role of the genus *Bifidobacterium*.

Fyderek *et al*. reported a lower abundance of *Bifidobacterium* in adolescents with IBD compared to their controls[184], while other studies have shown no reduction in the abundance of *Bifidobacterium* in patients with active CD[185,186]. Interestingly, an increase in the relative abundance of *Bifidobacterium* was reported in IBD adult patients compared to their healthy controls[187,188].

The present study found an association between fecal carriage and relative abundance of *B. longum* with biomarkers of EED. Fecal carriage of *D. formicigenerans* and biomarkers of EED were also significantly associated. *B. longum* carriage and relative abundance were associated with elevated fecal levels of AAT, MPO, NEO and high composite EED scores while carriage of *D. formicigenerans* was associated with normal MPO concentration and marginally associated with reduced chances of having high composite EED scores. The positive association between *B. longum* carriage and elevated biomarkers of EED is unusual considering that this bacterium has been largely shown to have beneficial effects in the human host. Nonetheless, this finding is consistent with data from IBD studies that showed an increase in the number of *Bifidobacterium* in IBD patients[188]. The positive association between *B. longum* and biomarkers of EED in the present study could be due to confounding by other factors such as breastfeeding and enteric infections, which were not assessed in the present study. Breastfeeding and enteric infections have been associated with elevated levels of MPO and AAT in Bangladeshi infants[161]. Nevertheless, age was adjusted for in the analyses and given the association between age and breastfeeding, adjusting for age in the analyses should have mitigated for confounding by breastfeeding. *D. formicigenerans* was associated with normal fecal MPO levels and reduced chances of having high composite EED scores in this study suggesting that this bacterium might be an indicator of normal gut health. However, multi-variable analysis showed that presence of *B. longum* negated the association between *D. formicigenerans* and composite EED score. This suggests *D. formicigenerans* association with composite EED score may be driven by reduced prevalence of *B. longum* in *D. formicigenerans* positive individuals, rather than a direct effect of *D. formicigenerans*.

5.3.3 F. prausnitzii and B. longum were associated with growth outcomes

The association between relative abundance of *F. prausnitzii, D. formicigenerans, B. longum* and ponderal (weight-related) growth has been shown before in murine studies. A study that compared fecal microbiota composition of mice transplanted with fecal samples from malnourished or healthy Malawian children reported a positive association between relative abundance of *B. longum, F. prausnitzii*, *D. formicigenerans* and weight gain in mice that harbored fecal samples from healthy children[76]. The present study found a negative association between intestinal carriage *of B. longum, F. prausnitzii* and anthropometric scores of ponderal growth (WAZ and WHZ). Intestinal carriage of *B. longum* and *F. prausnitzii* was associated with lower WAZ. Additionally, an increase in the relative abundance of *B. longum* was associated with increase in WHZ while an increase in the abundance of *F. prausnitzii* was associated with lower WHZ. To date there is no data on the association between presence or absence of *B. longum, F. prausnitzii* and ponderal growth in children but data exists on the association between relative abundance of genus *Bifidobacterium* and WHZ in children. Subramanian *et al.* found a lower abundance of the genus *Bifidobacterium* in Bangladeshi children with poor WHZ compared to children with better WHZ[168]. This finding is supported by data from this study that showed a positive relationship between relative abundance of *B. longum* and WHZ although the study by Subramanian *et al.* did not assess the associations at species level.

The negative associations between intestinal carriage of *B. longum, F. prausnitzii* and WAZ have not been reported before in human studies. This data suggests a potential role of these bacteria in ponderal growth. Murine studies have reported a positive association between the relative abundance of *B. longum, D. formicigenerans*, *F. prausnitzii* and weight gain, which is different from the data shown here. The inconsistency between these results suggests that these bacteria might have different effects on ponderal growth in mice and humans.

This study was limited by a number of factors. The use of commercial qPCR kits to describe prevalence of bacterial carriage limited the number of bacterial targets studied as primers and probes for other targets were not commercially available. The lack of standards in the commercial PCR kits precluded absolute quantification of each bacterium. The availability of data for a few demographic

variables limited the number of possible covariates that could have been considered in the analyses. Data analysis in the present study involved multiple comparisons, which could have had an effect on the interpretation of the results. Benjamini-Hochberg false discovery rate, Bonferroni correction and permutation are some of the methods used to correct for multiple comparisons. Further more refined analysis exploring multivariate models to examine collinearity in explanatory variables and examination of co-variance within the data are also methods by which the effect of individual predictors may be identified.

The use of a published bacterial qPCR assay that allowed relative quantification of each bacteria was one of the strengths of this study. Relative quantification of *B. longum* had a different relationship with WHZ compared with presence or absence of the bacterium suggesting that the association between *B. longum* and WHZ is dependent on the abundance and not just the presence of the bacterium.

5.3.4 Conclusion and future work

This study showed high prevalence of *F. prausnitzii* and *D. formicigenerans* in fecal samples from rural Malawian children. Carriage of these bacteria was more prevalent in older children compared to younger children. *B. longum* was found to be the least prevalent bacteria in the fecal samples and its carriage was associated with younger age. These results are consistent with existing data. *A. muciniphila*, which was present in almost half of the samples, was also shown to be associated with geographic location. Further investigations are needed to understand how the environment affects carriage of *A. muciniphila* and establish any subsequent effects in the context of EED and child growth. The study also found a relationship between *B. longum* or *D. formicigenerans* and biomarkers of EED. The positive relationship between *B. longum* and biomarkers of EED reported by this study is unusual as *B. longum* is widely thought of as beneficial to gut health. Therefore, there is need for further research to confirm the existence of such an association in this population. Additionally, future studies might sequence *B. longum* strains carried by children in this population to establish if there are any genetic differences with the strains that have been identified as beneficial in other populations as such data is lacking. Further studies should investigate if breastfeeding, hygiene, sanitation and other factors

confound or interact with the relationship between *B. longum* and biomarkers of EED.

This study also reported negative associations between intestinal carriage of *B. longum, D. formicigenerans, F. prausnitzii* (identified by qPCR) and ponderal growth in children from low-income settings for the first time. These findings are different from results of experimental studies in mice that indicated a positive association between relative abundance of these 3 bacteria and weight gain. As such, there is need for further studies to validate such associations in children. Future studies could also investigate the effects of absolute bacterial count on ponderal growth compared to presence or absence of a bacterium. Interestingly, this study found a positive association between relative abundance of *B. longum* and WHZ. This finding supports data reported previously in children from similar low-income settings.

6. Characterization of the gut microbiota in EED

6.1.1 Introduction

The human gastrointestinal tract is inhabited by more than 100 trillion microorganisms, which include bacteria, archaea and eukarya. This collection of microorganisms is referred to as the gut microbiota. More than 50 bacterial phyla are known to make up the gut microbiota with *Bacteroidetes* and *Firmicutes* the most dominant phyla[189]. Microbial colonization of the gut starts at birth. A healthy human fetus is said to develop in a microbe-free environment, however, depending on the mode of delivery, the neonate encounters various maternal microbes that make up the initial gut microbiota at birth. Vaginally delivered infants have been reported to acquire bacterial communities like their mother's vaginal microbiota dominated by *Lactobacilli* and *Prevotella* while infants born via Cesarean section were shown to acquire bacterial communities like those found on their mother's skin surface dominated by *Staphylococcus* and *Corynebacterium*[65]. Gestation age also has an influence on gut microbiota composition and development. Preterm infants have been reported to have intestinal microbiota dominated by *Proteobacteria* and *Enterobacteriaceae* compared to full-term infants[190].

The gut microbiota continues to develop during the breastfeeding period and changes in species abundance during the complimentary feeding period towards a more 'adult-like' gut microbiota, with nutrition and the environment being the primary drivers. Breastfed infants have a *Bifidobacterium* dominated gut microbiota[191] which shifts to *Bacteroidetes*, *Clostridium* and *Eubacteria* spp. during weaning[192]. De Fillipo *et al.* compared the gut microbiota of rural African children, consuming a diet rich in fiber and non-animal protein, to the gut microbiota of European children who consumed a diet high in animal protein, sugar, starch and fat and low in fiber. The gut microbiota of rural African children was dominated by *Actinobacteria* and *Bacteroidetes* while *Firmicutes* and *Proteobacteria* were more abundant in European children[88]. This finding emphasizes the effect of diet on gut microbiota.

Another study that was undertaken to investigate age-related changes in gut microbiota composition showed that the infant gut microbiota is less stable and less diverse compared to that of an adult, which is dominated by *Firmicutes*,

Bacteroidetes and *Actinobacteria*[64]. More evidence on the impact of age on the gut microbiota has been shown recently by Odamaki *et al.* who reported that gut microbiota diversity increased sequentially with age until one's twenties when it stabilized[68]. Nevertheless, the stability of the adult gut microbiota does change with age. A less diverse gut microbiota with reduced abundance of *Firmicutes* and an increase in *Bacteroidetes* has been reported in the elderly (between 65 and 96 years)[193].

Flint and Hooper suggest the gut microbiota plays a crucial role in regulating physiologic and metabolic processes, which include nutrient utilization, immune development, pathogen inhibition and maintenance of the intestinal epithelium integrity[69,70]. Intestinal bacteria ferment undigested carbohydrates to short-chain fatty acids (SCFAs) under anaerobic conditions; SCFAs are major sources of energy for the host therefore the gut microbiota helps in the maintenance of host's energy balance[70]. Emerging evidence from *in vitro* studies indicates that the gut microbiota may also be key in the degradation of nondigestible dietary polysaccharides. It has been suggested that *Firmicutes* may be key degraders of nondigestible dietary polysaccharides thereby making energy available for utilization by the host[194,195]. The gut microbiota also plays a role in immune development. Evidence from animal studies indicate that the gut microbiota is key in the development of lymphoid structure particularly in the gastrointestinal tract of germ-free mice[196-198]. Furthermore, the gut microbiota prevents invasion and/or suppresses growth of enteric pathogens.

One of the ways through which intestinal bacteria prevent growth of pathogens is by releasing bacteriocins which are bactericidal molecules. A study investigating the effect of a probiotic bacteria derived bacteriocin on protection against *Listeria* infection in mice showed protective effects of the bacteriocin against *Listeria monocytogenes* infection[166]. Intestinal bacteria also prevent colonization by enteric pathogens by producing pathogen-inhibitory metabolites. SCFAs, which are metabolites of carbohydrate fermentation by intestinal bacteria, have been shown to inhibit intestinal colonization by pathogenic bacteria. Acetate produced by *Bifidobacteria* was shown to protect mice against death induced by *E. coli* O157:H7[71] while depletion of butyrate-producing *Clostridia* was associated with expansion of pathogenic *S. typhimurium* in mice[199].

The gut microbiota may also help in promoting mucosal barrier function of the intestinal epithelium via mucus production. It has been reported that the thin mucus layer of germ-free mice was restored to the thickness of that of conventionally raised mice upon exposure to luminal bacterial products potentially suggesting that the gut microbiota contributes to mucus production[200].

The gut microbiota is therefore essential in maintaining normal gut physiological and metabolic processes. Perturbations of the gut microbiota composition and function have been associated with disease[201]. Inflammatory bowel disease (IBD) refers to two conditions, ulcerative colitis (UC) and Crohn's disease (CD), which are characterized by chronic gut inflammation. The specific cause of IBD is not well established however its etiology has been linked with a mixture of genetics, gut microbiome, immune responses and environment that trigger unexpected immune responses to gut flora in people with genetic susceptibility[202-205]. With regard to the associations between gut microbiota and IBD, *Actinobacteria* and *Proteobacteria* have been reported to be more abundant in individuals with both IBD phenotype and genotype compared to controls while *Firmicutes* have been shown to be more abundant in the controls[206]. A study that explored gut bacterial community differences between individuals with IBD, specifically CD, reported a lower abundance of *Faecalibacterium prausnitzii* and an increased abundance of *Escherichia coli* in individuals with CD compared to their healthy twins[207].

Changes in the gut microbiota structure have also been linked with EED etiology. EED is a subclinical condition of the gut characterized by partial villous atrophy, impaired enterocyte tight junctions and underlying chronic inflammatory responses. Like IBD, the specific cause of EED is not clearly established however its etiology has been associated with ingestion of enteric pathogens and toxins via contaminated food and water. This exposure changes gut microbiota composition and function resulting in intestinal inflammation and changes in the intestinal epithelium structure[47]. There is limited data suggesting associations between the gut microbiota and EED. Recently, Ordiz and colleagues used 16S rRNA gene sequencing of fecal samples from rural Malawian children to identify the gut bacterial community structure associated with moderate to severe EED defined by an L:M ratio test result ranging from 0.15 to 0.45. The study reported

a significant reduction in *Proteobacteria* in children with EED compared to children without EED while gut microbiota diversity was not different[59].

The primary objective of this section of study was to characterize the fecal microbiota defined by 16S rRNA gene sequencing and explore associations with biomarkers of EED in rural Malawian children.

6.1.2 Study design and analyses

This study utilized baseline demographic data and fecal samples collected by the MORDOR-morbidity cross-sectional survey, described in detail chapter 3. Briefly, demographic data (age, sex and geographic location) and fecal samples were collected by the MORDOR-Malawi morbidity cross-sectional baseline survey from 709 children who were randomly selected in 30 rural clusters of Mangochi district. The MORDOR-Malawi morbidity study also had subsequent surveys at 12 months and 24 months after the baseline survey where fecal samples were also collected. To characterize the gut microbiota in EED, 103 baseline fecal samples were selected from the 709 baseline fecal samples that were collected by the MORDOR-Malawi morbidity baseline survey. Selection of the 103 fecal samples was based on the availability of corresponding samples at the subsequent 12-month or 24-month surveys as changes in the gut microbiota overtime was among the objectives of the PhD project. Thus, the 103 baseline fecal samples were from children who were randomly selected by the MORDOR-Malawi morbidity cross-sectional surveys at more than one-time point (baseline and 12 months, baseline and 24 months and all 3 time-points).

Proteins were extracted from the fecal samples and used for the quantification of biomarkers of intestinal inflammation (NEO and MPO) and intestinal permeability and protein loss (AAT) by ELISA as described in chapter 3. Total, genomic DNA was extracted from the fecal samples, as described in chapter 3.1.8.4, and 515F-806R indexed primers were used to amplify the V4 region of the bacterial 16S rRNA gene. This was followed by 2x300bp paired-end sequencing of purified amplicons on the Illumina MiSeq platform as described in chapter 3.1.8.7. Reagent and non-template controls were extracted and sequenced according to the same procedure. Reads were processed using QIIME 2 software (version 2018.6)[139] as described in chapter 3.1.8.7.4. Briefly, 16S rRNA sequences were

de novo-clustered into OTUs at ≥ 97% identity. Taxonomy was assigned to the OTUs using a naïve Bayes classifier pre-trained on the SILVA 16S database[142] subsequently generating an OTU table. To exclude spurious OTUs, only bacterial OTUs identified to the genus level, with sequences more than 0.005% of the total number of sequences and a frequency of more than 0.01% in any sample were retained in the analyses.

Characteristics of children whose fecal samples were selected for analysis were compared to those of all enrolled children to determine if they differed. Median (25^{th} ,75^{th} quartile) concentration of individual fecal biomarkers of EED were calculated to determine distribution of the concentrations. Western cut-off values for each of the 3 biomarkers[53,161] were used to determine proportions of fecal samples with elevated biomarkers. Raw concentrations of the 3 biomarkers were combined to form a composite EED score as described in chapter 3. Shannon (H) and Simpson (D) diversity indices were calculated from the OTUs to indicate alpha diversity using the phyloseq R package[148]. The relationship between alpha diversity and individual biomarker concentration was initially examined using scatterplots and Pearson correlation was conducted to explore the relationship between alpha diversity and biomarker concentration where the scatterplots indicated a linear relationship. Significant relationships between alpha diversity indices and outcomes of interest were investigated using regression analyses adjusted for a set of covariates. To determine bacterial phylogenetic distance between samples, weighted and unweighted UniFrac distance matrices were calculated using the phyloseq R package. To compare bacteria community compositional differences between groups, PERMANOVA with 1000 permutation tests was run on weighted and unweighted UniFrac distances and principal coordinate analysis (PCoA) plots were used to visualize differences in UniFrac clustering by groups. Differential abundance analysis at the genus level was performed to identify OTUs differentially abundant in EED. Zero-inflated negative binomial regression analysis was conducted, adjusting for age and sex, to establish the relationship between the differentially abundant OTUs and biomarkers of EED.

6.2 Results

6.2.1 *Baseline characteristics of participants*

A total of 1704 children were randomly selected out of which 1090 were enrolled into the MORDOR-Malawi morbidity baseline survey. Out of the 1090 children enrolled, 709 participated in fecal sampling at the baseline survey for the study of antimicrobial resistance. Of the 709 fecal samples, 103 had corresponding samples collected at 12-months and 24-months surveys and therefore were selected for 16S rRNA sequencing and ELISA assays at baseline. Enrollment and selection of samples for analyses are shown in **Figure 3.1.3.2** of chapter 3.1.3. Age, sex and residential location of children whose fecal samples were included in analyses compared to all enrolled children are shown in **Table 6.2.1.1** below. Proportions of male children and children who resided in Chilipa, Makanjira and Mangochi were comparable between all enrolled participants and those whose fecal samples were included in this study. However, children whose fecal samples were included for analyses were younger (mean age of 22.95 months vs mean age of 29.59 months) compared to all enrolled participants. Additionally, a larger proportion of children included in this analysis resided in Monkey Bay and a smaller proportion in Namwera compared to all enrolled participants. Out of the 103 fecal samples that were selected, 68 were tested for AAT, 62 for NEO and 75 for MPO. A total of 60 fecal samples had ELISA results for all 3 biomarkers.

Table 6.2.1.1: Characteristics of all participants who provided fecal samples compared to those enrolled.

Participant characteristic	Children included for analyses	All enrolled children	P-value
Number of participants	103	1090	
Male sex N (%)	52 (50.98%)	487 (44.7%)	0.3
Mean (SD) age in months	22.95 (13.54)	29.59 (16.42)	1.292e-05
Geographic location N (%)			
Chilipa	22 (21.34%)	235 (21.6%)	0.99
Makanjira	27 (26.21%)	225 (20.6%)	0.23
Mangochi	17 (16.5%)	237 (21.7%)	0.26
Monkey Bay	31 (30.1%)	192 (17.6%)	0.003
Namwera	6 (5.83%)	201 (18.4%)	0.002

6.2.2 *Sequence reads characteristics and taxon abundance in the fecal samples*

A total of 103 fecal samples were processed for bacterial 16S rRNA gene sequencing. One sample consistently failed to amplify and could not be sequenced. All sequences for the 102 samples were subjected to post-sequencing quality filtering processes and all samples (n=102) were retained for analysis after the quality filtering processes. The final dataset generated a total of 1,685,792 reads with an average read depth per sample of 16,527±11,071. A total of 9 phyla, 16 classes, 25 orders, 43 families and 117 classified and 6 unclassified genera were identified from the sequence reads. Seven genera (*Faecalibacterium, Streptococcus, Bacteroides, Haemophilus, Subdoligranulum, Bifidobacterium and Escherichia-Shigella*) were present in about 62% of the samples and together they accounted for 46.4% of the whole bacterial community; *Faecalibacterium* was prevalent in 81% of the samples making it the

most prevalent genus*. Bifidobacterium* was prevalent in 51% of the samples and it had the highest number of reads, which accounted for 14.9% of the total reads. After rarefaction to 1000 reads with 1000 permutations, 69 genera belonging to 7 phyla, *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Tenericutes, Verrucomicrobia*, were retained. Out of the 7 identified phyla, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* were the most dominant and accounted for 64.23%, 13.8%, 8.9% and 6.5% of the total bacterial community respectively (**Figure 6.2.2.1**).

As part of quality control, qPCR data for the 4 bacteria described in chapter 5 (*B. longum, A. muciniphila, D. formicigenerans, F. prausnitzii*) and *Ruminococcus gnavus* (tested as detailed in chapter 3.1.8.5) was used to verify genera presence in the OTUs. **Table 6.2.2.1** shows the number of samples classified as positive for each of the specific bacterial species tested by qPCR and that were positive for the corresponding bacterial genera (OTU). Of the 47 samples that tested positive for *B. longum* in the specific qPCR, 44 had the genus *Bifidobacterium* present in the OTUs, 99 samples positive for *F. prausnitzii* in the qPCR, 88 had the genus *Faecalibacterium* present in the OTUs while only 14 of the 49 samples that tested positive for *A. muciniphila* in qPCR had their genus present in the OTUs (**Table 6.2.2.1**). A mock bacterial community that composed of *Hemophilus influenza, Moraxella catarrhalis* and *Staphylococcus epidermis* was also included as a positive control in all sequencing runs. Only reads corresponding to the 3 genera (*Hemophilus, Staphylococcus* and *Moraxella*) were obtained from the mock control sample. Reagent and non-template controls were processed together with the samples and these generated 21,550 reads. The major taxon present in these controls was *Streptococcus*.

Table 6.2.2.1 Verification of genera presence in OTUs by specific qPCR

Variable (n=102)	**Genus in OTU**	
qPCR target	Absent	Present
Bifidobacterium longum positive	3	44
Akkermansia muciniphila positive	35	14
Dorea formicigenerans positive	44	29
Faecalibacterium prausnitzii positive	18	81
Ruminococcus gnavus positive	20	3

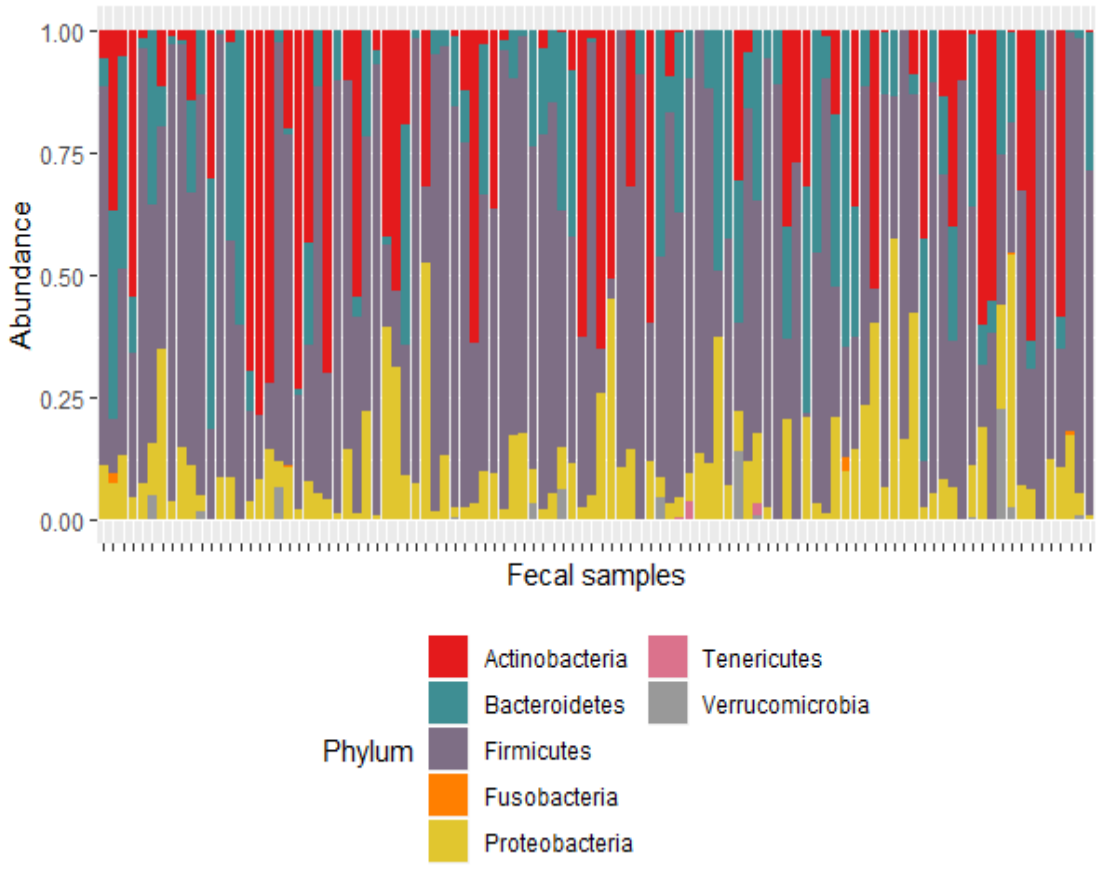

Figure 6.2.2.1 Relative abundance of major phyla found in fecal samples. *The proportion of total number of reads for each phylum in a sample represents phylum abundance after rarefaction to 1000 reads.*

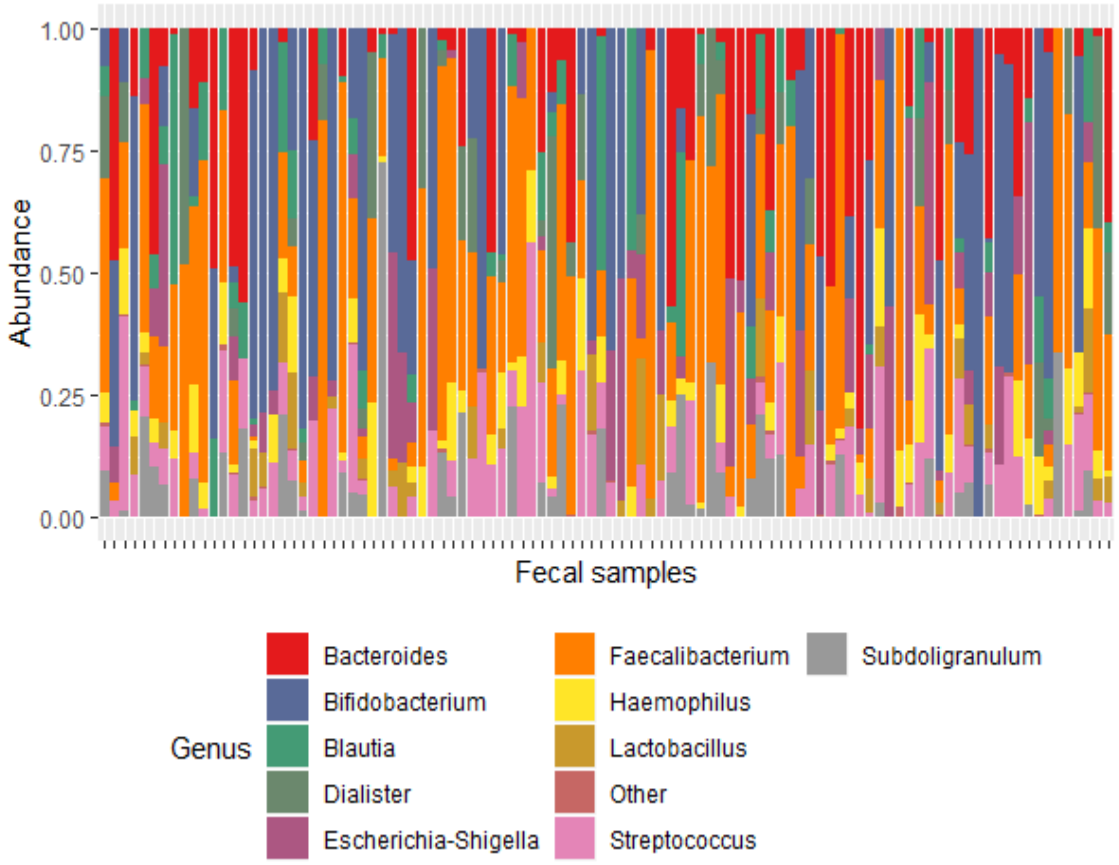

Figure 6.2.2.2 Relative abundance of major genera in fecal samples. *The proportion of total number of reads for each genus in a sample represents genus abundance after rarefaction to 1000 reads. The stacked bar plot only shows the 10 most abundant genera. The remaining genera have been grouped as "Other".*

6.2.3 *Effect of age on the fecal microbiota*

The effect of age on fecal microbiota composition and diversity in the samples was tested using the rarefied OTU dataset (comprised of 102 samples contributing 7 phyla and 69 genera). Simpson and Shannon diversity indices were calculated from the rarefied OTU data to indicate alpha diversity in the samples. Visualization of the distribution of Shannon and Simpson indices in all fecal samples showed a normal distribution for Shannon and a left-skewed distribution for Simpson index (**Figure 6.2.3.1**). The mean (SD) Shannon index was 1.72 (0.45) and median (25^{th}, 75^{th} quartile) Simpson index was 0.75 (0.66,0.82). Child age in months was categorized as 0-12, 13-24, 25-36 and 37-60. The differences in Shannon and Simpson distribution by age group were statistically tested using ANOVA and the non-parametric Kruskal-Wallis test respectively. There were significant differences in the distribution of both Shannon ($P<0.001$, Figure

6.2.3.2 A) and Simpson (P=0.004, **Figure 6.2.3.2 B**) by age. Both Shannon and Simpson indices were lower in fecal samples from younger children compared to older children, indicating increasing diversity with age.

Weighted UniFrac and unweighted UniFrac distances were calculated to determine bacterial phylogenetic distances between samples. PERMANOVA with 1000 permutation tests was used to statistically compare weighted UniFrac and unweighted UniFrac distances between age groups and PCoA plots were used to visualize differences in clustering. Age again had a significant effect on fecal microbiota as indicated by PERMANOVA outputs using unweighted and weighted UniFrac (R^2 =0.17, F=6.4, P=0.001 and R^2=0.29, F=12.8, P=0.001 respectively) and PCoA plots for both unweighted UniFrac and weighted UniFrac (**Figures 6.2.3.3** and **6.2.3.4**). Stacked bar plots were then plotted to visualize taxa that were different at phylum and genus levels between age groups (**Figure 6.2.3.5** and **6.2.3.6**). Fecal samples from younger children (0-12 months) were shown to have a higher abundance of *Actinobacteria* and lower abundance of *Bacteroidetes* (Kruskal-Wallis test P=0.01). At genus level, higher abundance of *Bifidobacterium* and a lower abundance of genus *Faecalibacterium* were observed in younger children (0-12 months) compared to the older age groups (Kruskal-Wallis test P=0.01).

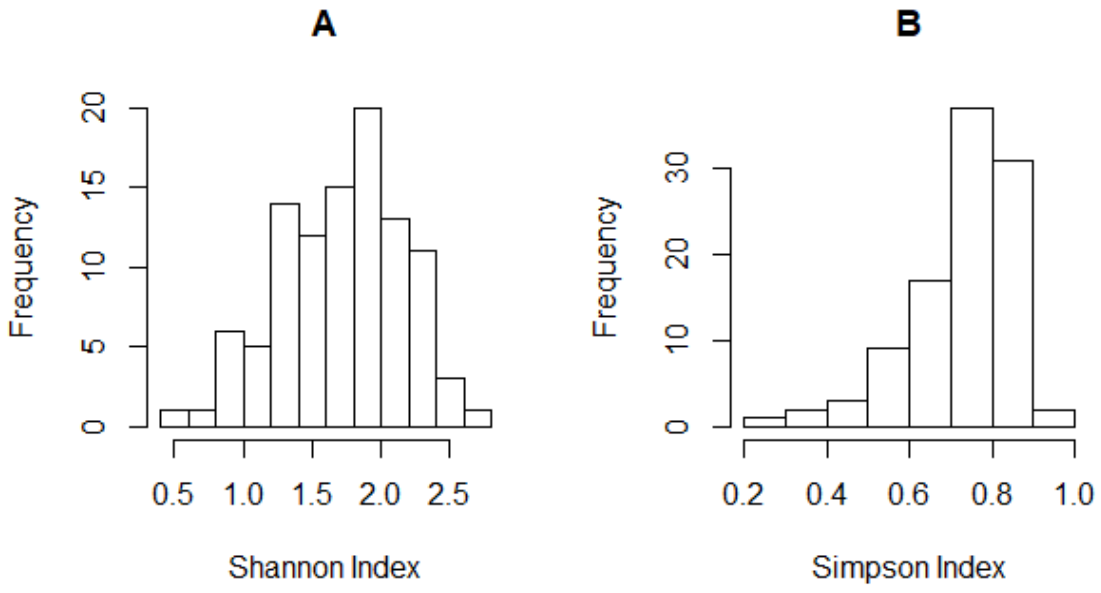

Figure 6.2.3.1 Shannon and Simpson diversity indices distribution for all the fecal samples included in final analysis.

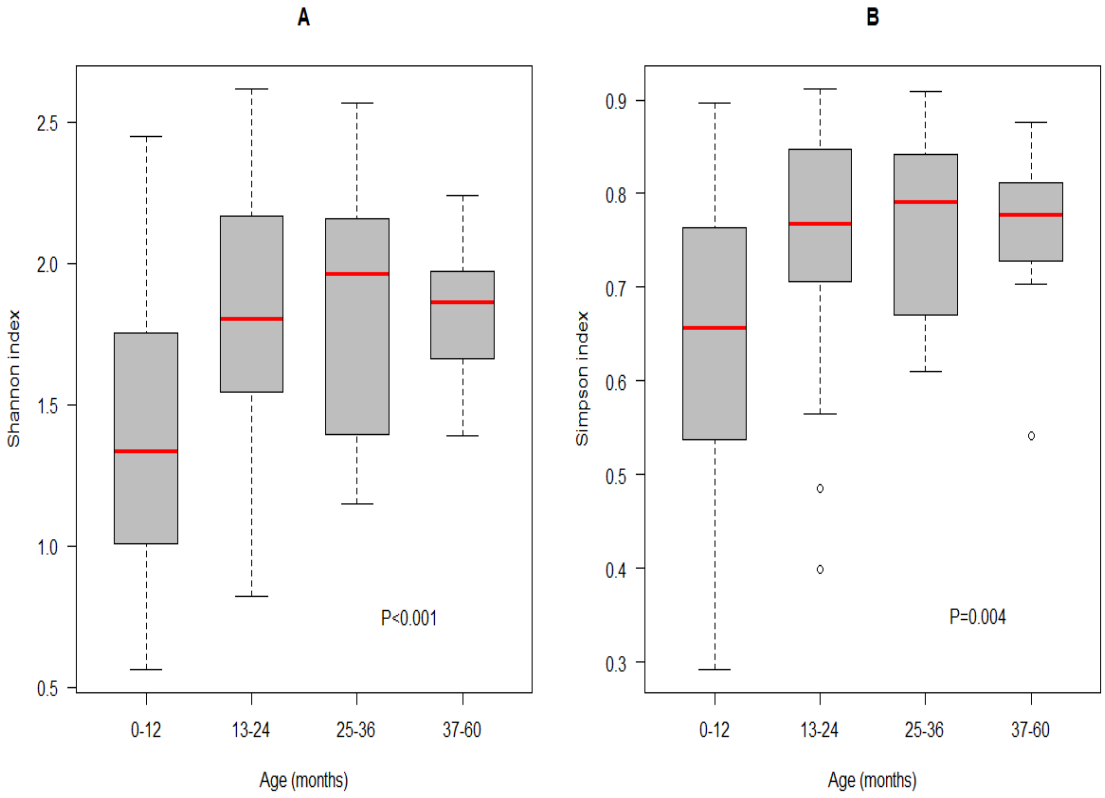

Figure 6.2.3.2 Comparison of (A) Shannon and (B) Simpson indices distribution between age groups. *P-value for Shannon index (P<0.001) was obtained from ANOVA while P-value for Simpson index (P=0.004) was obtained from the Kruskal-Wallis test.*

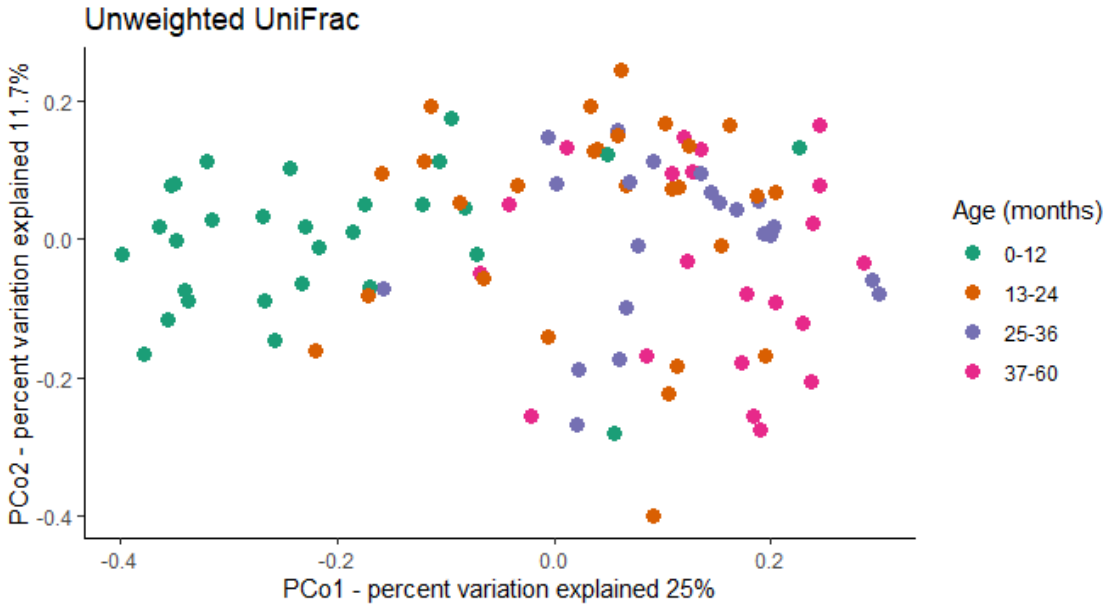

Figure 6.2.3.3 PCoA plot of unweighted UniFrac by age. *PCo1 explained 25% of total variation while PCo2 explained 11.7% of total variation. Children aged 0-12 months were clustered in the negative space of PCo1. Children aged >24 months were clustered in the positive space of PCo1. Children aged 13-24 months clustered between 0-12 and 37-60 age groups.*

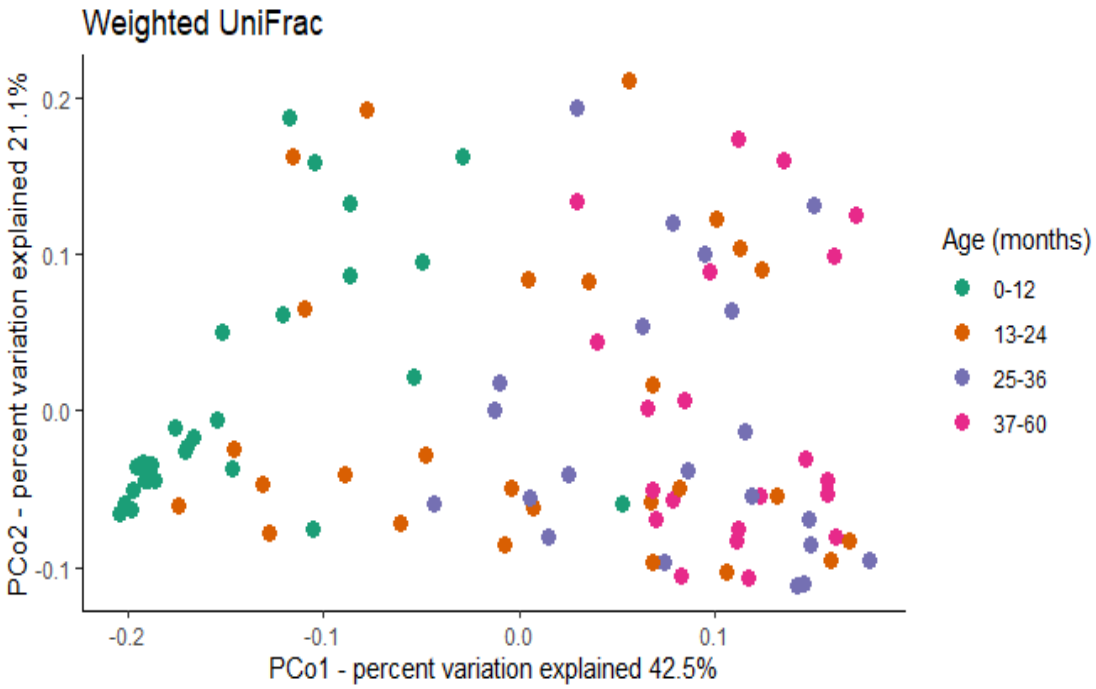

Figure 6.2.3.4 PCoA plot of weighted UniFrac by age. *PCo1 explained 42.5% of total variation while PCo2 explained 21.1% of total variation. Children aged 0-12 months were clustered in the negative space of PCo1. Children aged >24 months were clustered in the positive space of PCo1. Children aged 13-24 months spread across PCo1.*

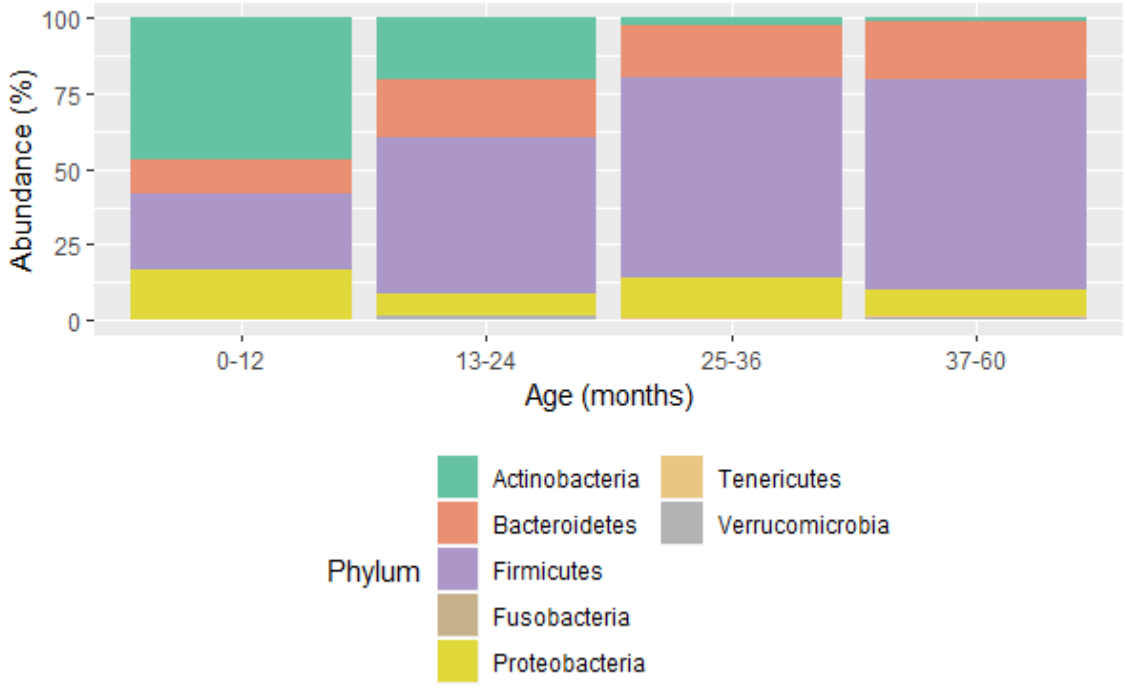

Figure 6.2.3.5 Relative abundance of phyla age.

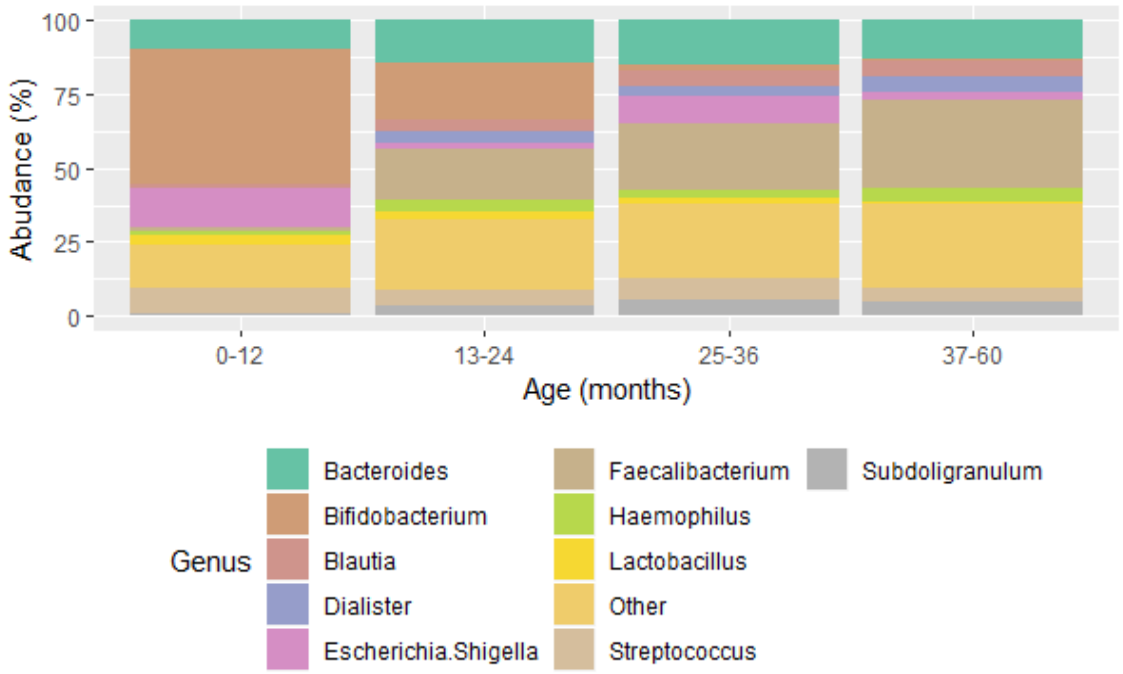

Figure 6.2.3.6 Relative abundance of genera by age. *The stacked bar plot only shows the 10 most abundant genera. The remaining genera were grouped as "Other".*

6.2.4 *Effect of sex on fecal microbiota*

The effect of sex on fecal microbiota diversity and composition in the samples was also tested. The Student's t-test showed no difference in Shannon distribution by sex (P=0.12) while the Wilcoxon-rank test showed no difference in Simpson distribution by sex (P=0.27).

Weighted UniFrac distances were then calculated to determine bacterial phylogenetic distances between samples by sex. Significant effect of sex on fecal microbiota composition was detected by PERMANOVA using both weighted UniFrac distances (R^2=0.04, F=4.3, P=0.002) and unweighted UniFrac (R^2=0.037, F=3.9, P=0.001). After adjusting for age however visualization by PCoA did not indicate prominent differences in clustering in male and female children for both unweighted and weighted UniFrac (**Figures 6.2.4.1** and **6.2.4.2).** Differences in taxa abundance by sex were then visualized at phylum and genus levels using stacked bar plots (**Figure 6.2.4.3** and **6.2.4.4)** and linear regression analysis, adjusting for age, was used to test for associations between sex and phyla or genera**.** Sex was not associated with relative abundances of phyla or genera (linear regression P>0.9).

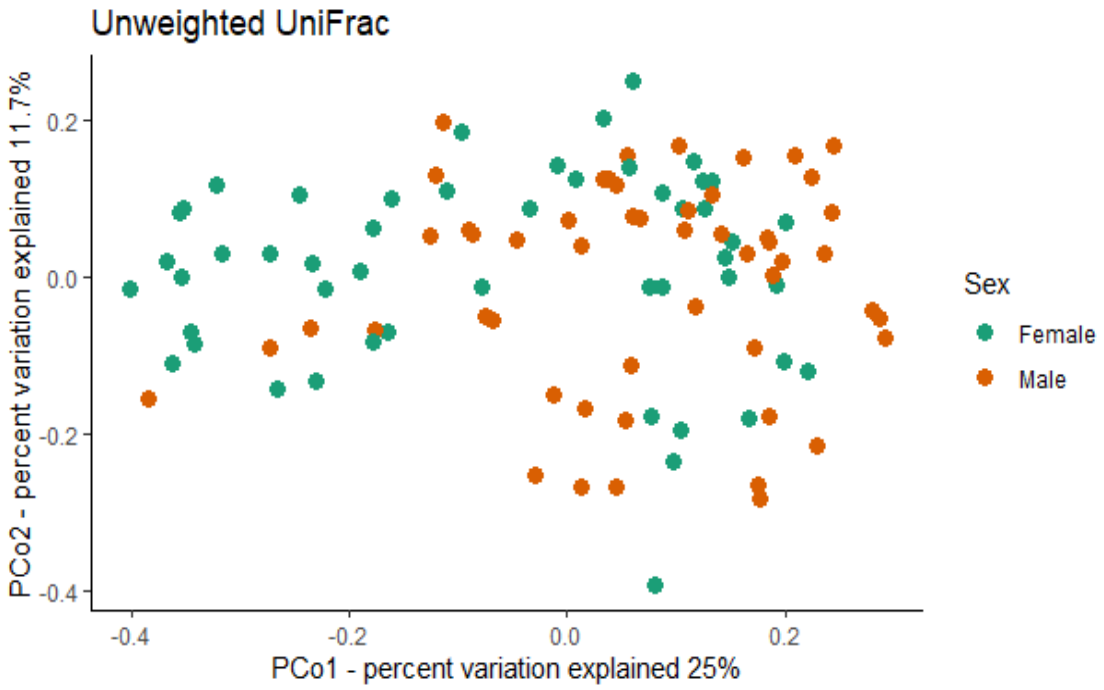

Figure 6.2.4.1 PCoA plot of unweighted UniFrac by sex. *PCo1 explains 25% of total variation while PCo2 explained 11.7% of total variation. Male children clustered more in the positive space of PCo1 compared to female children.*

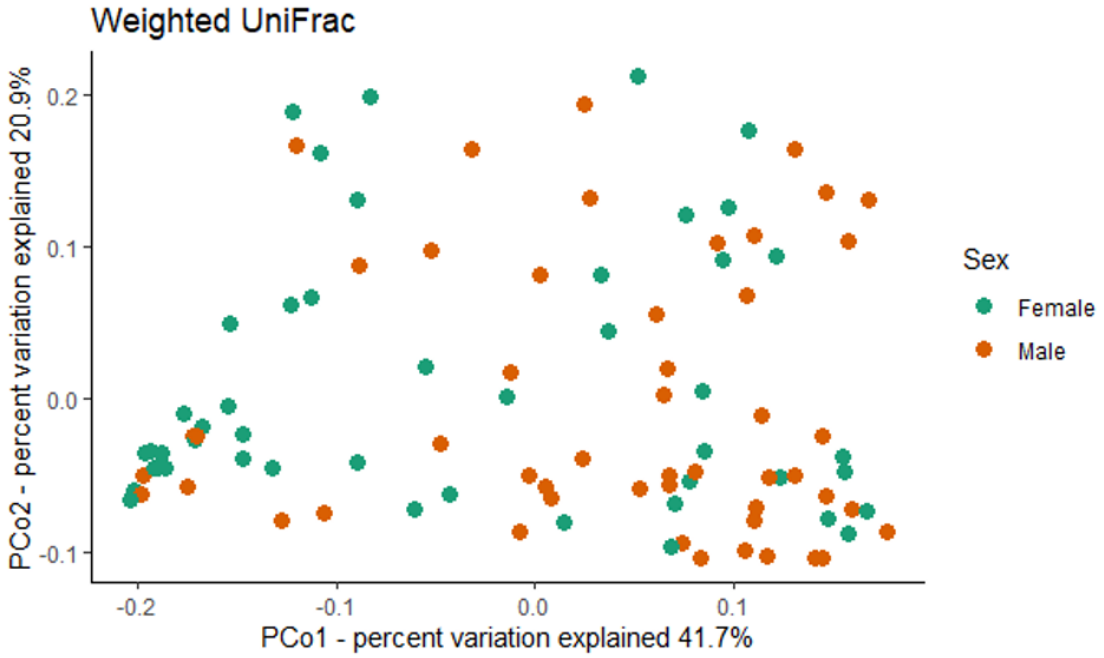

Figure 6.2.4.2 PCoA plot of weighted UniFrac by sex. *PCo1 explained 41.7% of total variation while PCo2 explained 20.9% of total variation. Male and female children did not cluster differently on either PCo1 or PCo2.*

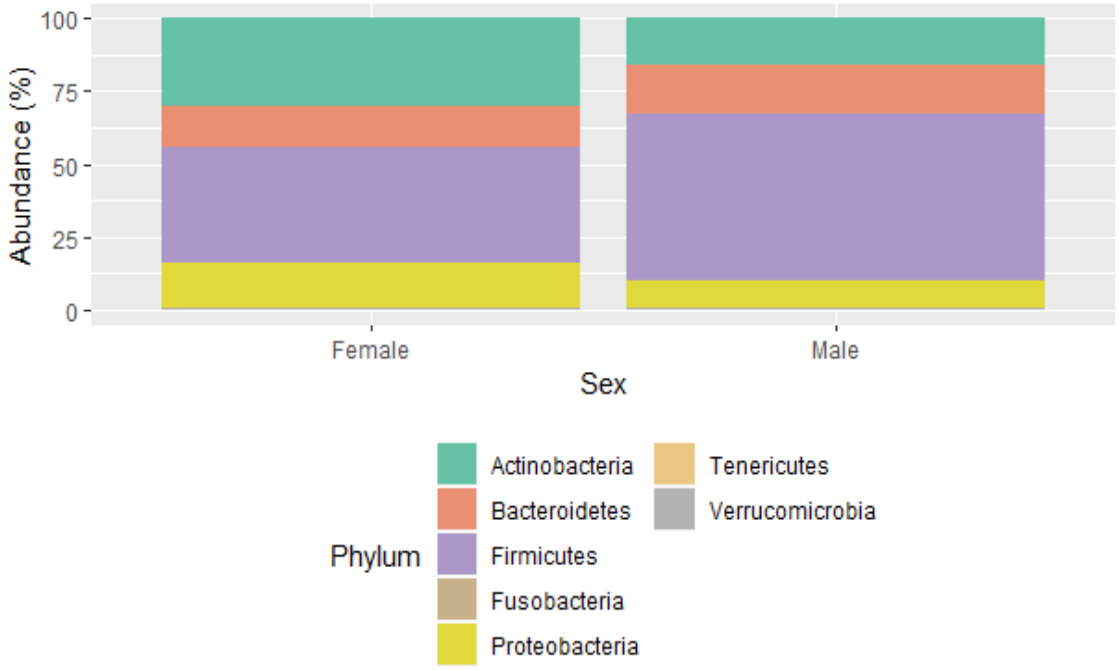

Figure 6.2.4.3 Relative abundance of phyla by sex

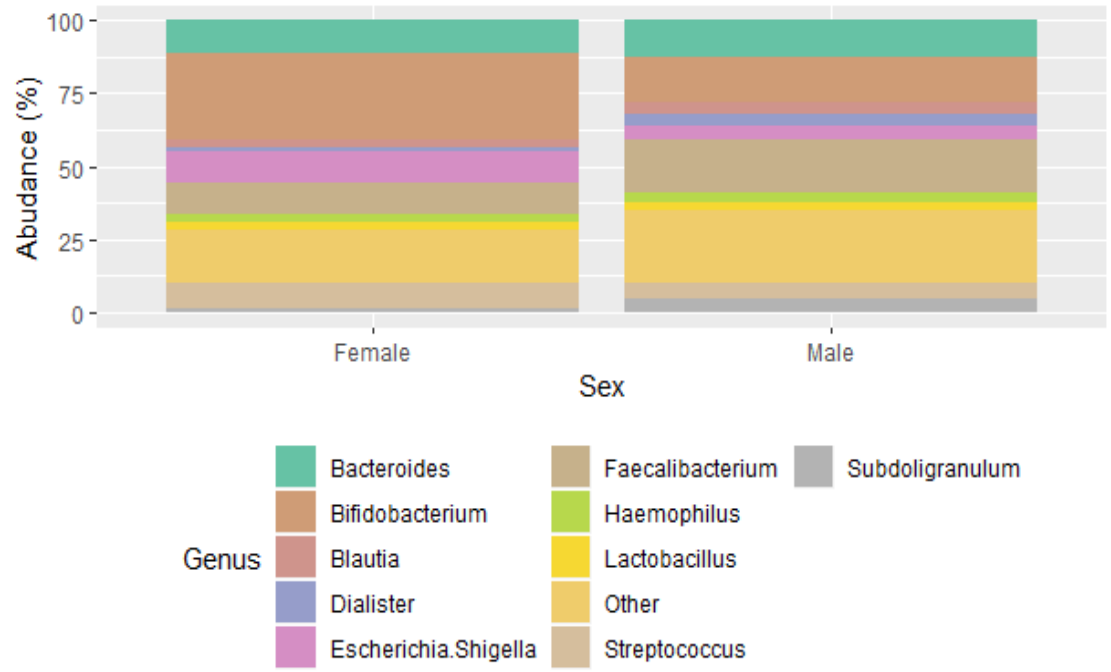

Figure 6.2.4.4 Relative abundance of genera by sex. *The stacked bar plot only shows the 10 most abundant genera. The remaining genera were grouped as "Other".*

6.2.5 *Effect of residential location on fecal microbiota*

The effect of participant's residential location on fecal microbiota diversity and composition in the samples was also explored. There were no differences in

Shannon distribution by participant's residential location as indicated by ANOVA (P=0.25). Likewise, the Kruskal-Wallis test showed no differences in Simpson distribution by residential location (P=0.13).

Weighted and unweighted UniFrac distances were then calculated to determine bacterial phylogenetic distances between samples. There were no significant effects of residential location on fecal microbiota composition detected by PERMANOVA using both unweighted (R^2=0.05, F=1.26, P=0.147) and weighted UniFrac (R^2=0.06, F=1.4, P=0.128).

6.2.6 *Associations between measures of fecal microbiota diversity and biomarkers of EED*

In this analysis, AAT, NEO, MPO and composite EED score described in chapter 4 were used as biomarkers of EED. Out of the 102 fecal samples that characterized the fecal microbiota by 16S rRNA sequencing, 68 had corresponding ELISA results for AAT, 75 for MPO, 62 for NEO and 60 had ELISA results for all the 3 biomarkers and therefore were included in the presented analyses. The relationship between Shannon or Simpson diversity indices and raw biomarker concentration was examined using scatter plots and tested using Pearson's correlation. Shannon index showed a linear relationship with all the 3 biomarkers (**Figure 6.2.6.1 A-C**) while Simpson showed a significant negative correlation with AAT and NEO but not MPO (**Figure 6.2.6.1 D-F**). Linear regression analyses were then conducted to model the relationship between Shannon or Simpson diversity and the individual biomarkers, adjusting for age and sex. Each of the 3 biomarkers was used in a linear regression model as a continuous outcome variable. There was no evidence of a relationship between Shannon diversity and AAT (P=0.14) or MPO (P=0.46) nor was there a relationship between Simpson diversity and AAT (P=0.09) or MPO (0.92). However, there were weak negative associations between Shannon diversity or Simpson diversity and NEO. An increase in Shannon diversity or Simpson diversity was associated with a reduction in NEO concentration [coefficient= -759, P=0.044, 95 % CI (-1496, -21) and coefficient= -2710, P= 0.047, 95% CI (-5386, -34) respectively].

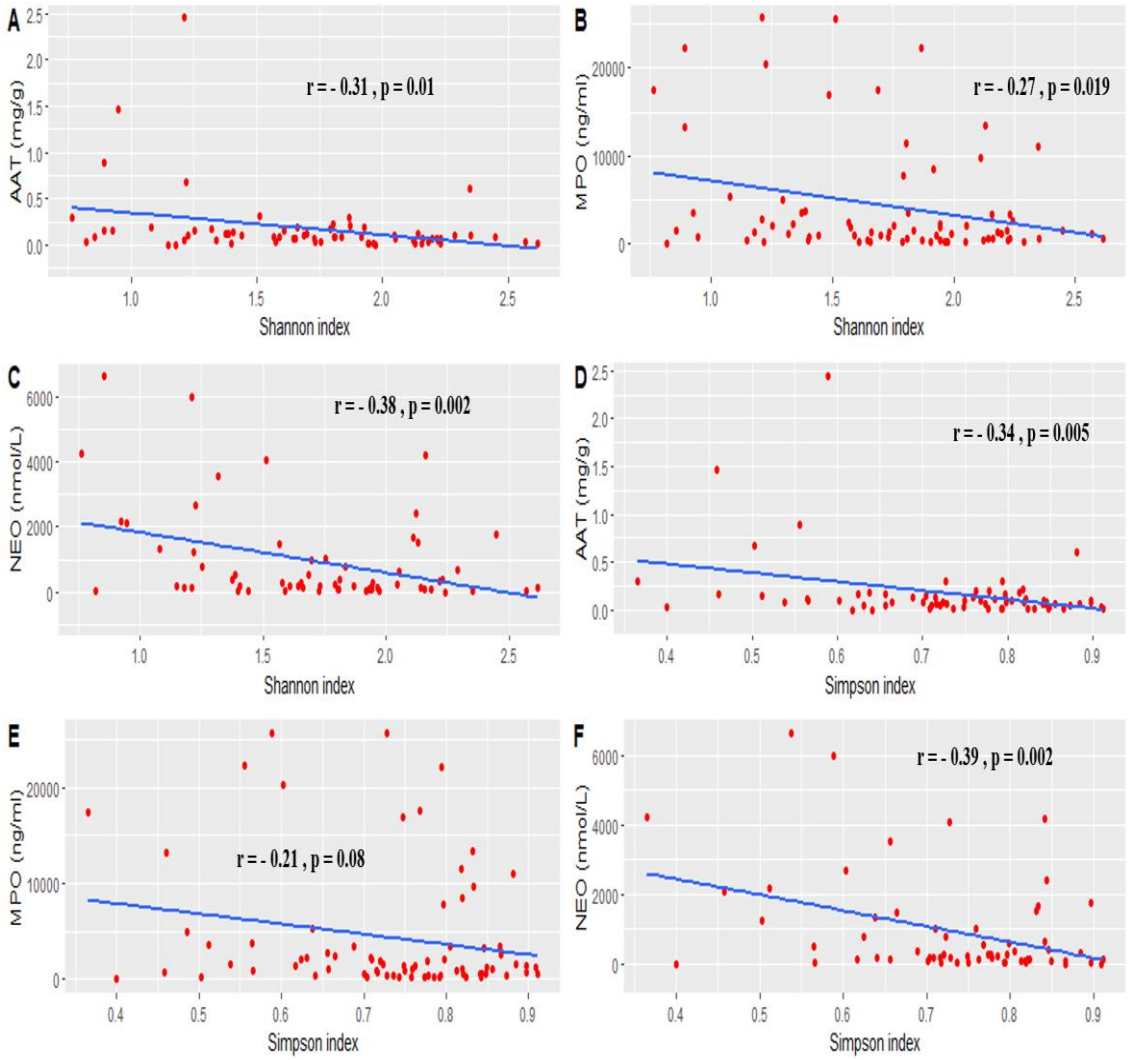

Figure 6.2.6.1 Scatterplots for the relationship between alpha diversity measures and individual biomarkers of EED. *(A) Shannon index and AAT, (B) Shannon index and MPO, (C) Shannon index and NEO, (D) Simpson index and AAT, (E) Simpson index and MPO, (F) Simpson index and NEO. The correlation coefficient values and P-values were obtained from Pearson's product-moment correlation.*

Of the 102 participants, 60 had ELISA results for all the 3 biomarkers from which the composite EED score could be calculated. Thus, data from these 60 participants was used to explore the associations between gut microbiota diversity and the composite EED score. The composite EED scores were categorized into low and high scores centered around the mean score (2.01). Logistic regression analysis, adjusted for age and sex, showed no association between Shannon or Simpson diversities and composite EED scores (P=0.14 and P=0.33 respectively).

6.2.7 *Associations between fecal bacterial community structure and biomarkers of EED*

Fecal biomarker concentration was then categorized as normal or elevated using the Western thresholds for AAT (≤0.27mg/g), NEO (≤70 nmol/L) and MPO (≤2000 ng/ml)[53,161,162]. Of the fecal samples included in this analysis, 11.8% (8/68) had elevated AAT concentration while 41.3% (31/75) and 82.3% (51/62) had elevated concentrations for MPO and NEO respectively. Bacterial community compositional differences between fecal samples with normal and elevated biomarker concentration were tested using PERMANOVA with 1000 tests. No differences in bacterial community composition between normal and elevated biomarkers were detected by PERMANOVA for each of the 3 biomarkers.

Differences in fecal microbiota composition between low and high composite EED score were also tested using PERMANOVA that was run on unweighted and weighted UniFrac distances adjusting for age and sex. PCoA plots were used to visualize UniFrac clustering between the two groups. There were significant differences in bacterial community composition between fecal samples with low and high composite EED scores indicated by PERMANOVA for unweighted ($F=2.53$, $R^2=0.04$, $P=0.011$) and weighted UniFrac ($F=3.35$, $R^2=0.05$, $p=0.017$) and shown in PCoA plots (**Figures 6.2.7.1** and **6.2.7.2**).

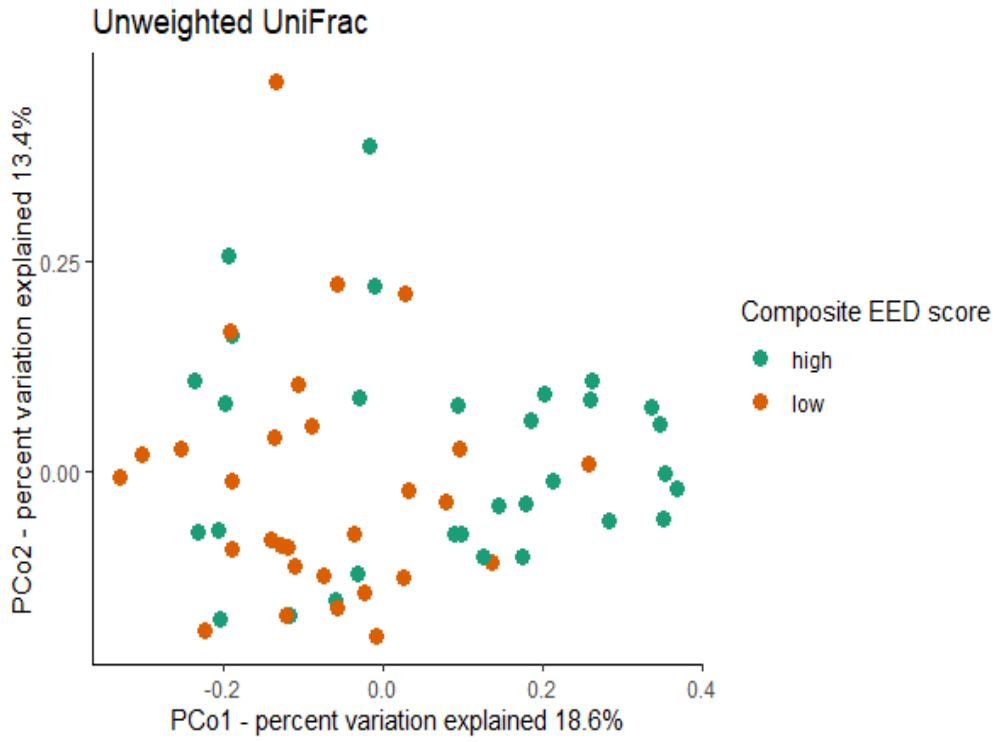

Figure 6.2.7.1 PCoA plot of unweighted UniFrac by composite EED score. *PCo1 explained 18.6% of total variation while PCo2 explained 13.4% of total variation. Individuals with low composite EED scores clustered more on the negative space of PCo1 while individuals with high composite EED score clustered more on the positive space of PCo1.*

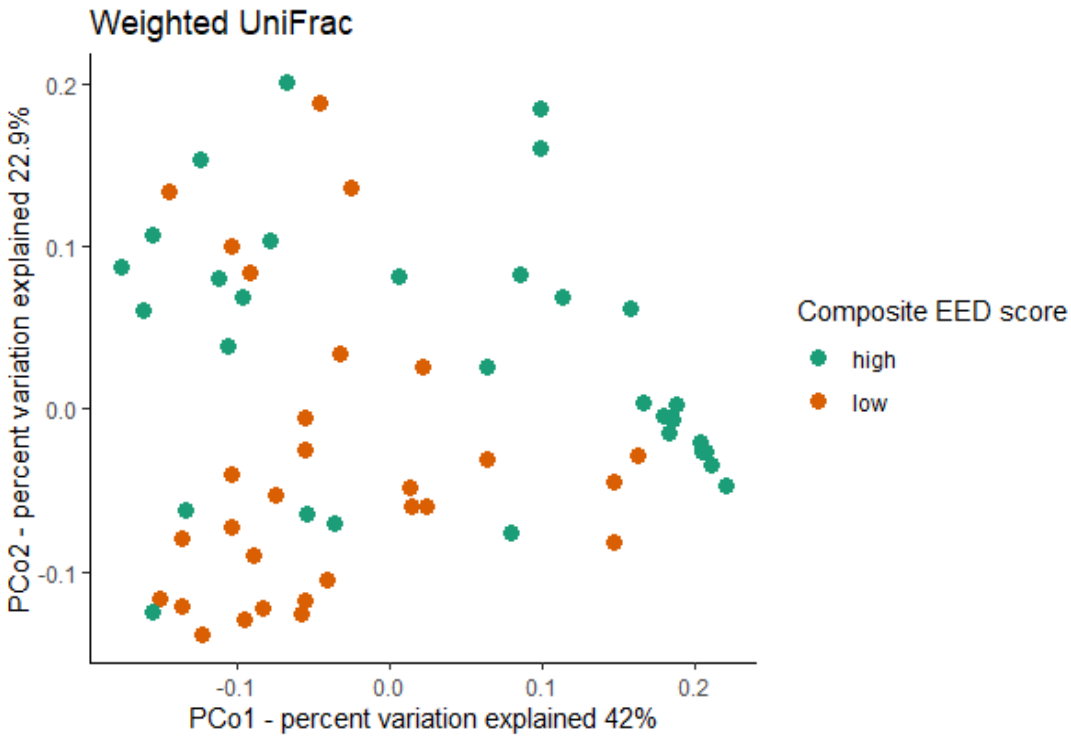

Figure 6.3.7.2 PCoA plot of weighted UniFrac by composite EED score. *PCo1 explained 42% of total variation while PCo2 explained 22.9% of total variation. Individuals with low composite EED scores clustered more on the negative spaces of PCo1 and PCo2.*

Differential abundance analysis at genus level was then conducted to determine OTUs that were differentially abundant between low and high composite EED score. Three genera (*Succinivibrio*, *Butyrivibrio and Enterococcus*) were differentially abundant in fecal samples with high composite EED score compared to fecal samples with low composite EED score (**Table 6.2.7.1**). A zero-inflated negative binomial regression analysis was conducted to explore the relationship between the differentially abundant OTUs and the composite EED scores, adjusting for age and sex. Out of the 3 differentially abundant genera, only 1 genus (*Succinivibrio*) was associated with composite EED score after adjusting for age and sex. An increase in the abundance of *Succinivibrio* was associated with 0.14 reduced odds of a having a high composite EED score compared to low composite EED score (**Table 6.2.7.1**).

Table 6.2.7.1 Genera differentially abundant in fecal samples with high composite EED compared to low composite EED scores.

Genus	log_2Fold Change	lfcSE	P-value[a]	padj[b]	OR[c] (95%CI)	P-value[c]
Succinivibrio	-26.3	2.6	5.24e-24	2.25e-22	0.14 (0.08,0.23)	1.51e-41
Butyrivibrio	-25.6	2.9	5.02e-19	1.08e-17	3.52 (0.68,18.16)	0.791
Enterococcus	23.5	2.9	7.72e-16	1.11e-14	1.82 (0.25,1.2)	0.619

[a]Denotes unadjusted P-value while [b]denotes p-value obtained after adjusting for multiplicity with Benjamin-Hochberg P-value correction.

OR=odds ratio.

CI=confidence interval.

[c]Denotes OR (95%CI) and P-values obtained with zero-inflated negative binomial regression. (conditional model) after adjusting for age and sex.

lfcSE = log fold change Standard Error.

6.3 Discussion

The present study explored associations between the fecal microbiota and biomarkers of EED in rural Malawian children. Sequencing of the bacterial 16SrRNA gene and quantification of the biomarkers of EED in fecal samples allowed the assessment of associations between fecal microbiota diversity or composition and biomarkers of EED (individually or combined). Verification of the presence of genera in OTUs by species-specific qPCR showed concordance between qPCR and V4-16S rRNA sequencing for *Bifidobacterium* and *Faecalibacterium*, whereas *Ruminococcus, Dorea* and *Akkermansia* differed between the two assays. The variations between the two assays suggest a difference in sensitivity; qPCR is more sensitive than V4-16S rRNA sequencing. Additionally, the quality control results indicate that the accuracy of V4-16S rRNA sequencing might be dependent on the abundance of a genus. *Bifidobacterium* and *Faecalibacterium*, which were well detected by the two assays, were found to be high in abundance in the fecal samples included in this study compared to the other 3 genera.

The study found no association between fecal microbiota diversity and individual fecal concentration of AAT and MPO. Similarly, there was no association between fecal microbiota diversity and the composite EED score. However, the study found a negative association between fecal microbiota diversity and fecal concentration of NEO. A comparison of bacterial community composition by individual biomarker concentration showed no significant differences, but significant differences in fecal microbiota composition were found when the individual biomarkers were combined to form a composite EED score suggesting perturbations of overall gut microbiota structure in EED. Secondary analyses on the effects of age, sex and residential location showed that age, but not sex or residential location, was associated with fecal microbiota. The findings on the relationship between age and gut microbiota are consistent with previous studies[64,68].

6.3.1 *Effect of age on microbiota diversity and microbiota composition*

Age-related changes on fecal microbiota diversity and composition have been reported previously. Increased age has been associated with increased fecal microbiota diversity and changes in fecal microbiota composition[208]. The present study divided age into 4 categories from 1 to 59 months of age to compare fecal microbiota diversity and composition across age groups. Microbiota diversity was higher in older age groups compared to younger children. There was a marked difference in microbiota diversity seen among children from 1 to 36 months of age. The study also found differences in microbiota composition across age groups. Fecal samples from younger children were shown to have a higher abundance of phylum *Actinobacteria* and lower abundance of phylum *Bacteroidetes*. At genus level, higher abundance of *Bifidobacterium* and a lower abundance of genus *Faecalibacterium* were seen in younger children compared to the older age groups. These findings are consistent with trends reported by recent longitudinal studies[64,68]. The increase in diversity and changes in intestinal microbiota seen with the different age groups are also consistent with changes that have been reported during the transition from breast feeding to weaning[191,192].

6.3.2 *Effect of sex on fecal microbiota diversity and composition*

Associations between sex and gut microbiota have been reported in adults. One study that assessed associations between sex and gut microbiota in European adults reported a higher abundance of *Bacteroides* in males compared to females[209]. However, there is limited data on the associations between sex and the gut microbiota in infants and young children. One recent study that explored the associations between sex and gut bacteria found higher abundance of *Lactobacillus*, a dominant genus in the vaginal microbiota, in females than males in early life[210]. The present study compared the fecal microbiota of male and female children aged not more than 5 years. Fecal microbiota diversity and composition were not different in fecal samples from male compared to female children suggesting that sex does not impact on gut microbiota in this group of children.

6.3.3 *Associations between the fecal microbiota and EED*

In the present study, alpha diversity was shown to have no relationship with fecal levels of AAT or MPO, however a negative association between alpha diversity and NEO, a biomarker of intestinal inflammation, was found. The study found no significant differences in fecal microbiota composition between normal and elevated fecal biomarker concentration.

To date, there is no data available supporting a relationship between fecal microbiota diversity and intestinal inflammation in children at risk of developing EED. However, available evidence from inflammatory and autoimmune diseases suggest that increased intestinal microbiota diversity is an indication of a healthy status while a decrease in intestinal microbiota diversity is associated with disease[211,212]. Additionally, there is data from IBD studies in North American pediatric patients suggesting an association between intestinal inflammation seen in severe UC and reduced intestinal microbiota diversity[213]. These results are consistent with the present finding that alpha diversity is negatively associated with NEO, however, UC and EED are very different conditions that manifest differently thus the findings from the present study need to be further tested in larger EED studies. The current study did not find any association between fecal microbiota diversity and MPO, another biomarker of intestinal inflammation, nor were there any bacterial community differences between individuals with normal and elevated biomarkers of intestinal inflammation. Interestingly, the study showed significant differences in the fecal microbiota composition between low and high composite EED score. This finding supports the suggestion that no single biomarker is sufficient to indicate EED[161,162].

A study by Ordiz *et al*. that aimed to characterize the bacterial community structure in children with EED, as measured by L:M ratio test, reported significant differences in the composition of fecal microbiota of children with and without EED. At the phylum level, the study reported a significantly higher relative abundance of *Proteobacteria* in children without EED compared to children with EED while genera *Succinivibrio, Klebsiella and Clostridium_XI* were found to be reduced in EED[59]. These results are in part consistent with findings reported by the present study. Similar to findings by Ordiz *et al*., the present study found an association between increased abundance of the genus *Succinivibrio,* of phylum *Proteobacteria,* and reduced EED scores. In the bovine rumen, *Succinivibrio*, a

gram-negative anaerobic bacterium, has been associated with fiber-degradation[214]. Also, high abundance of this bacterium has been shown in fecal microbiota of rural African children, consuming a diet rich in fiber and non-animal protein[88] emphasizing the role of this bacterium in fiber degradation. Fiber degradation by anaerobic bacteria produces SCFAs that are major sources of energy for the host. SCFAs have also been shown to inhibit intestinal colonization by pathogenic bacteria[71,199]. Therefore, the significance of *Succinivibrio* in EED should be investigated further.

One of the limitations of this study included the use of Western values to define cut-off limits for normal or elevated biomarker concentration. This limitation is not unique to this study; the lack of reference values for the biomarkers of EED in children living in low-income settings has also been cited by other similar studies[161,162]. Current thresholds used by this and other published studies are reference values in European adults with IBD. However, the lack of reference values for the biomarkers of EED in children living in low-income settings makes the Western values the only available choice; there is a need for large-scale studies to define reference values of the biomarkers of EED in children living in resource-limited settings. The adoption of the Western reference values in this study might have undermined the magnitude of inflammation and also affected the plausible associations with the fecal microbiota, however, no associations between fecal microbiota and the uncategorized concentration values were found.

6.3.4 Conclusion and future work

This study suggested an association between intestinal microbiota diversity and intestinal inflammation, measured by fecal NEO, in children at risk of EED; increased fecal microbiota diversity was associated with a reduction in intestinal inflammation. However, the study did not find any significant associations between gut diversity and another marker of intestinal inflammation nor were there any differences in fecal microbiota composition between normal and elevated biomarkers. There is a need for large-scale studies to further test the relationship between microbiota diversity and intestinal inflammation in EED and investigate if the reported relationship between microbiota diversity and intestinal inflammation is a result of gut microbiota dysbiosis or vice versa. The study did

not find profound bacterial compositional differences between fecal samples with and without indications of EED, however, the study reported a finding that is consistent with a recent study in Malawian children. The genus *Succinivibrio,* which was reported by another study to be reduced in EED, was associated with composite EED scores. This bacterium has been associated with fiber-degradation and has recently been shown to be more abundant in children consuming a diet rich in fiber in low-income settings. Future studies need to investigate the role of this bacterium in EED. Such future studies could also explore the role of metabolites produced by such bacteria in preventing EED in children. Large-scale studies, conducted in multiple low-income settings, would be valuable in defining thresholds for the biomarkers of EED in children most at risk of developing this condition.

7. Effect of azithromycin on fecal microbiota

7.1.1 Introduction

The use of antibiotics has been shown to have growth-promoting effects in animals and humans. Early animal studies comparing animals given prophylactic antibiotics in their feed versus untreated animals reported growth-promoting effects of antibiotics[100-102]. Infants exposed to antibiotics in the first year of life have been reported to have higher chances of being overweight later in childhood compared to unexposed infants[215]. Prophylactic antibiotics and antibiotics given for infection have also been shown to promote weight gain in pre-pubertal children living in low-income countries[103,104] but the mechanism through which this happens is not known. One explanation for the effect of antibiotics on growth could be due to their ability to clear enteric pathogens or modulate the intestinal microbiota[105,106].

The impact of antibiotics on enteropathogens is well documented and the impact of antibiotics on the gut microbiota composition and development in mice and humans has been described. A study by Nobel and colleagues showed that beta-lactams or macrolides altered intestinal microbiota composition and delayed microbiota maturation in mice[216]. In that study, mice were treated with beta-lactams or macrolides during the second, fourth and fifth week of life and fecal samples were collected in between the treatments from the treated mice and their controls to determine short-term effects of antibiotics on the intestinal microbiota. The study reported reduction in alpha diversity a week after the initial treatment and a reduction in the abundance of *Bacteroidetes* after the third treatment in mice that were treated with beta-lactams or macrolides compared to their controls. Also, a comparison of overall gut microbiota composition between antibiotic-treated mice and their controls showed significant differences in microbiota composition between the two groups.

Other studies have also documented a reduction in gut microbiota diversity and changes in gut microbiota composition in the human population as a short-term effect of antibiotics on the gut microbiota. A study that examined microbiota diversity and composition in adults before and immediately after a 7-day course of antibiotic treatment reported a significant reduction in microbiota diversity and a shift from *Faecalibacterium* to *Bacteroides* as the most dominant genus[217]. A

recent study that investigated the short-term effects of antibiotics on the gut microbiota of infants reported a reduction in alpha diversity and changes in microbiota composition after antibiotic treatment[218]. Here, the intestinal microbiota, of children randomized to a 3-day course of azithromycin or placebo, was characterized at baseline and 14 days after treatment. The study showed a decrease in microbiota richness and a decrease in the relative abundance of *Proteobacteria* and *Verrucomicrobia* in fecal samples of treated children compared with the control group. Taken together, the above findings indicate short-term effects of antibiotics on gut microbiota diversity.

The long-term impact of antibiotics on the gut microbiota is not well established as available evidence is mixed. A study by Korpela *et al.* (detailed previously in chapter 1.8) showed that the antimicrobial effect of macrolides on the fecal microbiota in early childhood is long-term[127]. Here, a reduction in microbial richness in fecal samples from treated children compared to control samples was detected 12-24 months after treatment. Also, a significant decrease in the relative abundance of *Actinobacteria* and increased abundance of *Bacteroidetes* and *Proteobacteria* in fecal samples from children who had used macrolide antibiotics within 6 months of sample donation compared to controls was reported. However, another study by Wei *et al*. did not find any long-term differences in gut microbiota diversity between antibiotic-treated and untreated children[219]. Here, children aged 12-36 months were prescribed a 3-day course of azithromycin or placebo and the gut microbiota was characterized in fecal samples collected from each child at 4 years of age. The study reported no significant differences in alpha diversity (measured by observed richness and Shannon index) between the treatment and placebo groups. Additionally, there were no OTUs that differed significantly between groups. These inconsistent findings mean the long-term effects of macrolides on the gut microbiota are unclear.

Azithromycin is a broad-spectrum, macrolide antibiotic characterized by a long intra- and extra-cellular half-life. Its use is indicated in the treatment of atypical pneumonia, skin and soft tissue infections and sexually transmitted infections[110]. Community-wide mass azithromycin treatment is a core pillar of the WHO-recommended approach to control ocular infection with the intracellular bacterium *Chlamydia trachomatis*, the causative agent of trachoma[111]. Studies conducted

in trachoma endemic areas provide evidence that mass azithromycin treatment also reduces child morbidity and mortality[113-117].

The current study assessed the long-term impact of azithromycin treatment on the fecal microbiota of Malawian children. The study investigated the impact of up to 2 or 4 biannual azithromycin treatments on the diversity and composition of intestinal microbiota.

7.1.2 Study design and analyses

This study utilized demographic data and fecal samples collected by the MORDOR-Malawi morbidity study, described in detail in chapter 3. Briefly, MORDOR-Malawi morbidity randomized children in communities to azithromycin treatment or placebo at baseline and collected fecal samples cross-sectionally at baseline (n=709), 12 months post 2 biannual azithromycin administration (n=689) and 24 months post 4 biannual azithromycin administrations (n=873) to investigate the effect of carriage of antimicrobial resistant bacteria. A subset of fecal samples from 103 children, who were sampled, by chance, at two-time points were selected for the present study to investigate the long-term effect of azithromycin treatment on the gut microbiota.

Fecal samples were processed as described in chapters 3 (sections 3.1.8.4 and 3.1.8.7) and 6. V4-16S rRNA gene sequencing was conducted and Shannon (H) and Simpson (D) diversity indices were calculated from the OTUs to indicate alpha diversity. Differences in alpha diversity distribution between groups were tested using parametric (Student's t-test or ANOVA) or non-parametric tests (Wilcoxon-rank or Kruskal-Wallis tests). Weighted and unweighted UniFrac distance matrices were calculated to determine the bacterial phylogenetic distance between samples and were used to compare bacteria community compositional differences between groups in PERMANOVA analysis.

7.2 Results

7.2.1 Participant characteristics

From the children who participated in baseline fecal sampling, 54 also participated in sampling at 12 months. The results from these samples are referred to as the "baseline-12-month dataset". Fifty-five children participated in

baseline sampling and sampling at 24 months and their results are referred to as the “baseline-24-month dataset”. Within the baseline-12-month dataset, 30 children received placebo while 24 received azithromycin treatment. Within the baseline-24-month dataset, 30 children received placebo while 25 received azithromycin treatment. Baseline characteristics of all children in the azithromycin and placebo arms are shown in **Table 7.2.1**. For the baseline-12-month dataset, age, sex and Simpson diversity index were comparable between azithromycin and placebo arms but taxa relative abundance and Shannon diversity index were higher at baseline in azithromycin-treated children compared to those in the placebo arm. For the baseline-24-months dataset, all characteristics (age, sex, taxa relative abundance and alpha diversity) were comparable between azithromycin and placebo groups.

Table 7.2.1 Baseline characteristics of participants for the baseline-12months and baseline-24 months datasets.

	Treatment arm	
Participant characteristics	Azithromycin	Placebo
Baseline-12 months dataset		
Number of participants	24	30
Child male sex N (%)	14 (58.3%)	15 (50%)
Mean (SD) age, months	27 (14)	24.3 (15.4)
Median (25^{th} ,75^{th} quartile) taxa relative abundance	0.65 (0.54,0.77)	0.56 (0.49,0.64)
Median (25^{th} ,75^{th} quartile) Shannon diversity index	2.4 (1.9,2.5)	2 (1.6,2.2)
Median (25^{th} ,75^{th} quartile) Simpson diversity index	0.87 (0.78,0.89)	0.81 (0.73,0.85)
Baseline-24 months dataset		
Number of participants	25	30
Child male sex N (%)	13 (52%)	16 (53.3%)
Mean (SD) age, months	18.3 (10.6)	19 (10.8)
Median (25^{th} ,75^{th} quartile) taxa relative abundance	0.58 (0.51,0.66)	0.52 (0.45,0.65)
Mean (SD) Shannon diversity index	1.85 (0.47)	1.93 (0.55)

Median (25th ,75th quartile) Simpson diversity index	0.78 (0.67,0.83)	0.8 (0.74,0.84)

7.2.2 Up to 4 biannual azithromycin treatments did not have any long-term effect on fecal microbiota diversity

The baseline-12-month dataset was used to explore the effect of up-to 2 rounds of azithromycin on fecal microbiota diversity. Out of the 24 fecal samples originating from the 12-month survey in the azithromycin arm, 13 were from children who received 2 rounds of azithromycin, 10 from children who received 1 round and 1 sample from a child who did not receive any azithromycin. At 12 months, a comparison of fecal microbiota diversity between azithromycin and placebo arms showed no differences in alpha diversity, measured by Shannon diversity index [median (25th, 75th quartile) of 2.15 (2,2.42) vs 2.22 (1.7,2.37), Wilcoxon's test P=0.71] and Simpson diversity index [median (25th, 75th quartile) of 0.83 (0.78,0.87) vs 0.81 (0.78,0.87), Wilcoxon's test P=0.72). Similarly, there were no differences in Shannon diversity (Wilcoxon's test P=0.88) or Simpson diversity (Wilcoxon's test P=0.99) distribution between baseline and 12-month fecal samples in the azithromycin arm regardless of the number of treatment rounds received.

To assess the effect of up to 4 rounds of biannual azithromycin treatment on fecal microbiota diversity, the baseline-24-month dataset was used. Out of the 25 fecal samples originating from the 24-months survey in the azithromycin arm, 9 were from children who received 4 rounds of azithromycin treatment, 4 from children who received 3 rounds, 6 from children who received 2 rounds, 3 from children who received 1 round and 3 from children who did not receive treatment. Comparison of fecal microbiota diversity between azithromycin and placebo arms at 24 months showed no differences in Shannon diversity [mean (SD) of 2.2 (0.47) vs 2.2 (0.4), Student's t-test P=0.97] and Simpson diversity [median (25th, 75th quartile) of 0.82 (0.78,0.87) vs 0.83 (0.77,0.88), Wilcoxon's test P=0.91). Comparison of Simpson diversity index distribution between baseline and 24-month fecal samples in the azithromycin group showed no differences between the two time-points. Shannon diversity tended to be higher in the 24-month fecal samples compared to baseline samples [mean (SD) of 2.2 (0.46) vs 1.97 (0.36),

Student's t-test P=0.057)], which was not significant after adjusting for age and sex (linear regression, P=0.192).

7.2.3 Azithromycin treatment was associated with changes in fecal microbiota composition at 24-months follow-up

At 12 months, PERMANOVA analysis using unweighted (R^2 =0.01, F=0.69, P=0.85) and weighted (R^2 =0.01, F=0.47, P=0.89) UniFrac distances detected no differences in overall fecal microbiota composition between azithromycin-treated children and children who received placebo. Similarly, there were no differences in microbiota composition between the baseline and 12-month fecal samples in the azithromycin group based on weighted (R^2 =0.03, F=1.39, P=0.21) and unweighted (R^2 =0.03, F=1.35, P=0.14) UniFrac distances nor were there differences in microbiota composition by number of azithromycin treatments received by the 12-month fecal collection (PERMANOVA based on weighted UniFrac, R^2 =0.05, F=1.01, P=0.42 and PERMANOVA based on weighted UniFrac, R^2 =0.03, F=0.75, P=0.76). Relative abundance showed increasing trends in *Prevotella* and *Ruminococcaceae* at 12 months compared to baseline and reduced trends of *Streptococcus* and *Bacteroides* at 12 months compared to baseline (**Figure 7.2.3.1**). At 24 months, PERMANOVA analysis did not detect any differences in overall fecal microbiota composition between azithromycin and placebo groups based on weighted (R^2 =0.005, F=0.24, P=0.97) and unweighted (R^2 =0.015, F=0.79, P=0.73) UniFrac distances and comparison of microbiota composition by number of treatment rounds within the azithromycin arm showed no effect based on weighted UniFrac distances (PERMANOVA, R^2 =0.24, F=1.54, P=0.095) and unweighted UniFrac distances (PERMANOVA, R^2 =0.07, F=0.83, P=0.81). However, a comparison of fecal microbiota composition between baseline and 24-month fecal samples within the azithromycin group showed significant differences based on unweighted UniFrac distances (PERMANOVA, R^2 =0.1, F=5.09, P=0.001 and PCoA, **Figure 7.2.3.2 A**) and weighted (PERMANOVA, R^2 =0.1, F=5.1, P=0.001 and PCoA, **Figure 7.2.3.2 B**).

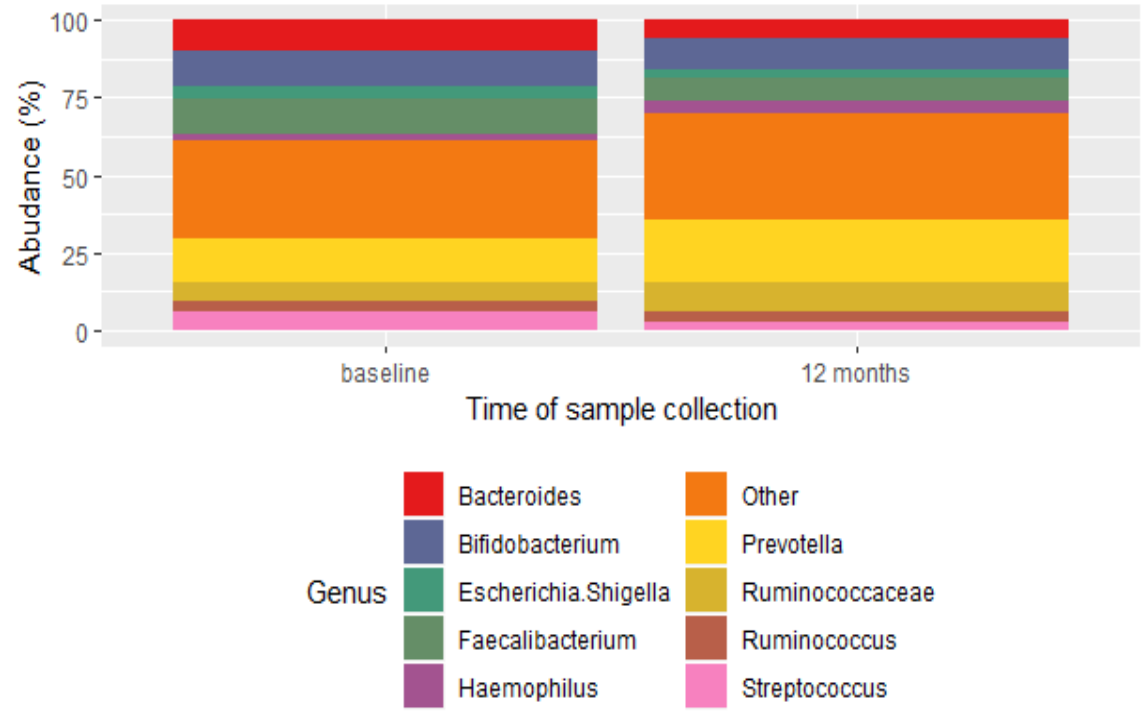

Figure 7.2.3.1 Relative abundance of genera in baseline and 12-month fecal samples within the azithromycin group. *Only shows the 9 most abundant genera are shown. The remaining genera were grouped as "Other".*

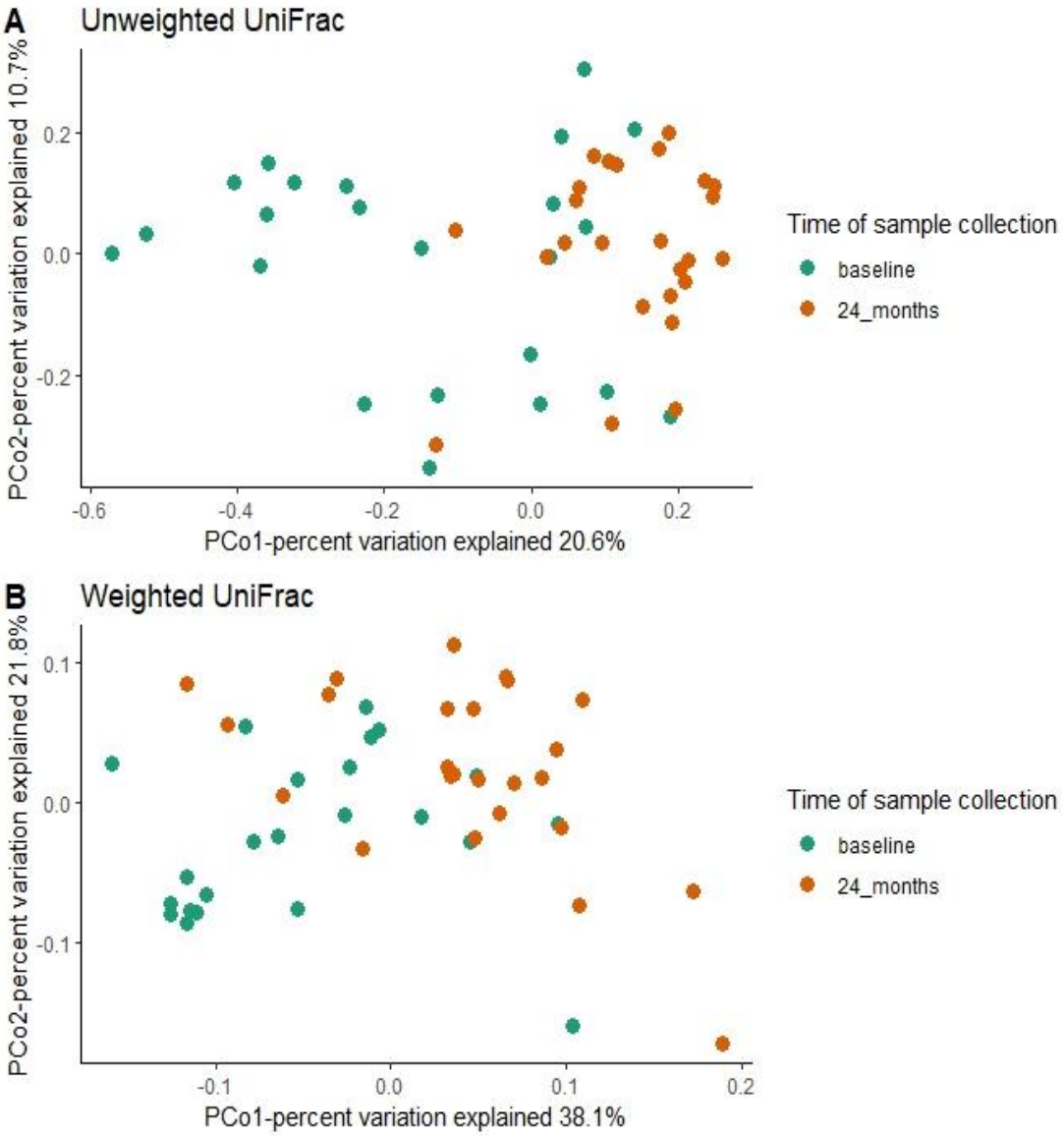

Figure 7.2.3.2 Overall fecal microbiota composition differences between baseline and 24-month fecal samples in the azithromycin arm. *(A) PCoA plot of unweighted UniFrac distances and (B) PCoA plot of weighted UniFrac distances. In both A and B, PCo1 explained the most variation and baseline fecal samples clustered in the negative space of PCo1 while 24-months fecal samples clustered in the positive space of PCo1.*

Taxa relative abundances of fecal samples collected at baseline and 24 months, within the azithromycin and placebo groups, were compared and visualized using stacked bar plots at phylum and genus levels. Crude comparisons of taxa relative abundance at the phylum level showed a higher relative abundance of *Bacteroidetes* in the 24-month fecal samples compared to baseline samples [median (25^{th},75^{th} quartile) of 0.38 (0.31,0.45) at 24 months vs 0.21 (0.08,0.40) at baseline, Wilcoxon P=0.004] while *Actinobacteria* was shown to be more abundant in fecal samples collected at baseline in both the azithromycin [median (25^{th} ,75^{th} quartile) of 0.08 (0,0.47) at baseline vs 0.01 (0,0.02) at 24 months,

Wilcoxon P=0.014] and placebo groups [median (25^{th} ,75^{th} quartile) of 0.14 (0,0.31) at baseline vs 0.0001 (0.00,0.01) at 24 months, Wilcoxon P=0.001] (**Figures 7.2.3.3 A and 7.2.3.4 A**). At the genus level, *Bacteroides* was more abundant at baseline compared to 24 months in the placebo group (**Figure 7.2.3.4 B**). *Bifidobacterium* and *Streptococcus* were shown to be more abundant in fecal samples collected at baseline compared to 24-month fecal samples while genera *Prevotella* and *Ruminococcaceae* were more abundant in fecal samples collected at 24 months compared to baseline fecal samples in both treatment groups (**Figures 7.2.3.3 B and 7.2.3.4 B**). Age and sex-adjusted generalized linear mixed models (with the participant as a random effect) were then used to assess the longitudinal differences in the relative abundance of individual genera in fecal samples of children who received azithromycin or placebo. The analyses showed no significant differences in the relative abundance of *Bifidobacterium, Streptococcus*, *Ruminococcus*, and *Ruminococcaceae* between baseline and 24 months in both treatment arms, however, the interaction between time of sample collection and azithromycin treatment was associated with 60% decreased odds of *Streptococcus* abundance at 24 months (odds ratio of 0.4, P=0.008) compared to baseline in the azithromycin arm. Additionally, azithromycin treatment was associated with 30% increased odds of relative abundance of *Prevotella* at 24 months (odds ratio of 1.3, P=0.05) compared to baseline within the azithromycin arm. Adding time of sample collection as an interaction term did not make any significant difference. Age and sex-adjusted generalized linear mixed models (with the participant as a random effect) showed no longitudinal differences in the placebo arm.

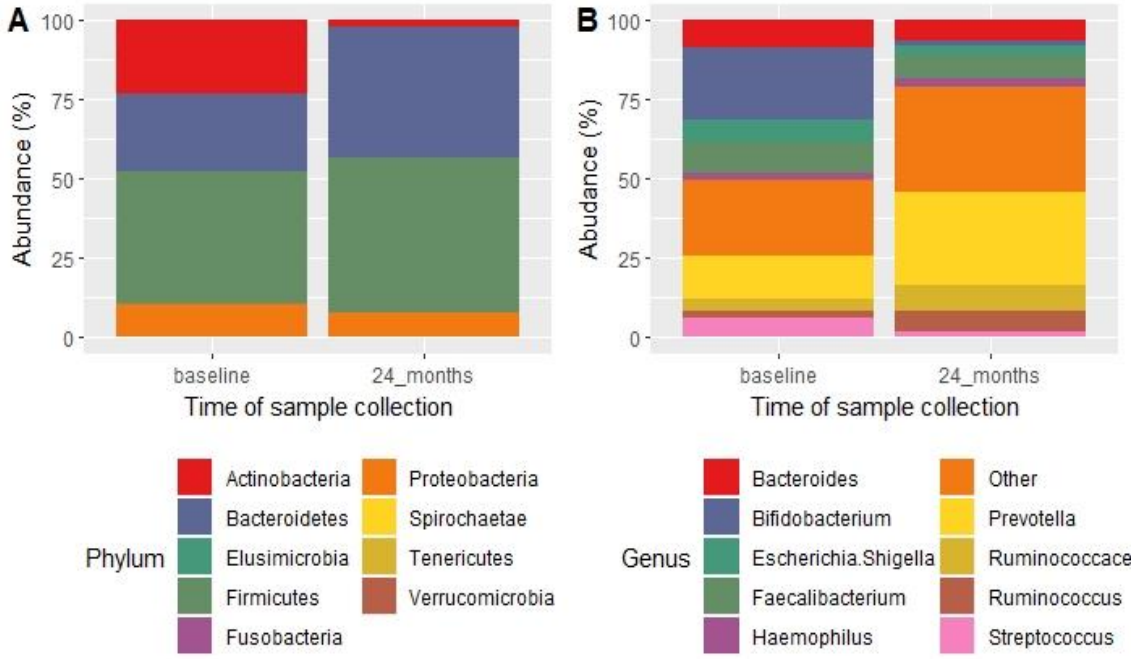

Figure 7.2.3.3 Relative abundance of phyla (A) and genera (B) in baseline and 24-month fecal samples within the azithromycin group. *Figure 7.2.3.3B only shows the 9 most abundant genera. The remaining genera were grouped as "Other".*

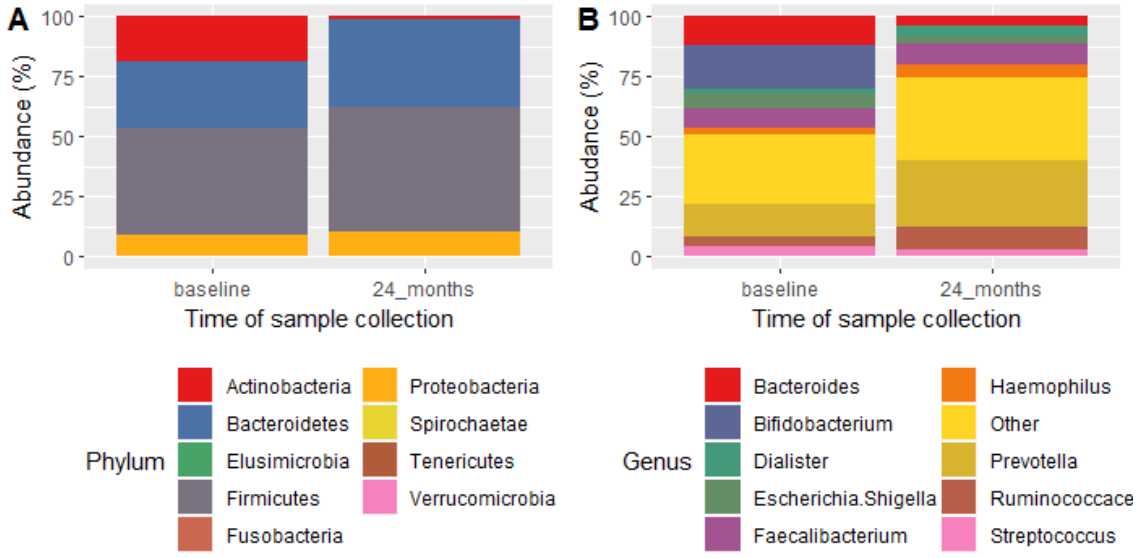

Figure 7.2.3.4 Relative abundance of phyla (A) and genera (B) in baseline and 24-month fecal samples within the placebo group. *Figure 7.2.3.4B only shows the 9 most abundant genera. The remaining genera were grouped as "Other".*

7.3 Discussion

This study assessed the long-term impact of mass azithromycin treatment on the intestinal microbiota. There were no differences in gut microbiota diversity or composition between azithromycin and placebo arms at 12 or 24 months. However, age and sex-adjusted longitudinal assessments of the gut microbiota within the azithromycin arm showed marginal differences in gut microbiota composition between baseline and 24-months samples. A higher abundance of *Prevotella* and a lower abundance of *Streptococcus* were observed at 24-months follow-up compared to baseline samples in the azithromycin arm suggesting long-term azithromycin-related changes in gut microbiota composition. Age and sex-adjusted longitudinal assessments in the placebo arm did not show any significant differences.

7.3.1 Azithromycin treatment was associated with changes in gut microbiota composition at 24-months follow-up

The present study assessed the impact of up to four biannual azithromycin administrations on intestinal microbiota diversity and composition. This study did not find any effect of up to four biannual azithromycin treatments on microbiota diversity, which is consistent with data by Wei *et al*.[219], although the follow-up period in the present study was shorter. This study did however find weak associations between azithromycin treatment and microbiota composition. Azithromycin treatment was weakly associated with increased abundance of *Prevotella* at 24-months follow-up compared to baseline within the azithromycin arm while no differences were detected in the placebo arm. While this finding has not previously been reported, it is consistent with a recent study that investigated the effect of azithromycin on gut microbiota composition and reported an increased abundance of *Bacteroidetes,* the phylum to which *Prevotella* belongs*,* in children who received macrolides within 6 months preceding samples collection compared to untreated controls. *Prevotella* is a gram-negative commensal bacterium found on the mucosal sites of the respiratory tract, the gut, and the oral cavity. Reduced abundance of this bacterium has been associated with Crohn's disease in pediatric patients[220]. Therefore, increased abundance of *Prevotella* at 24-months following azithromycin treatment suggests that long-term azithromycin treatment potentially increases the abundance of a bacterium beneficial to the gut. Azithromycin treatment was also associated with reduced

abundance of *Streptococcus* at 24-months follow-up compared to baseline when the time of sample collection, within the same individual, was considered. The reduction in *Streptococcus* abundance at 24-months follow-up suggests time-dependent antimicrobial effects of azithromycin.

One of the limitations of this study was availability of fewer fecal samples compared to the total number of children enrolled by MORDOR-Malawi morbidity at baseline. The fecal samples included in the analyses were selected based on the availability of corresponding baseline and 12 months or baseline and 24-month fecal samples, which might have been prone to selection bias as a smaller number of children at baseline participated in fecal sampling compared to the total number of children enrolled.

7.3.2 Conclusion and future work

This study did not find any long-term impact of up to four biannual azithromycin treatments on alpha diversity, however, longitudinal assessment of the gut microbiota showed weak long-term associations between azithromycin treatment and the abundance of defined taxa that had not been demonstrated before; increased relative abundance of *Prevotella* and reduced relative abundance of *Streptococcus* were associated with azithromycin treatment. The association between azithromycin treatment and relative abundance of *Prevotella* is consistent with a previous study showing an increase in abundance of the phylum *Bacteroidetes*, to which *Prevotella* belongs. Future studies could determine whether the increased abundance of *Bacteroidetes* or *Prevotella* spp. after long-term exposure to azithromycin is a result of selective macrolide resistance and if there are any subsequent effects on gut health.

8. Discussion

8.1 No evidence of association between fecal biomarkers of EED and growth outcomes in rural Malawian children

In this study, biomarkers of intestinal inflammation (NEO and MPO) and protein-losing enteropathy or intestinal permeability (AAT) were quantified in fecal samples and their associations with growth outcomes were assessed. Two-thirds of the children had raised NEO levels and one-third of the children had raised fecal MPO concentration. Intestinal permeability or protein-losing enteropathy, measured by fecal AAT, was prevalent in approximately 16% of children. The biomarkers showed no correlation between themselves and all decreased with increasing age. Additionally, children who resided in Namwera had higher levels of AAT and MPO compared to children who were resident in Mangochi, Monkey Bay, Mankanjira or Chilipa. None of the biomarkers, assessed individually or combined, showed a relationship with poor growth outcomes. The differences in the proportion of children with raised NEO and children with raised MPO, both of which are markers of intestinal inflammation, in addition to lack of correlation between biomarkers of intestinal inflammation and intestinal permeability, suggests that intestinal inflammation in EED is triggered by independent mechanisms and that intestinal inflammation does not always lead to intestinal permeability. The lack of a relationship between intestinal inflammation or permeability and growth outcomes may suggest that the impact of EED on growth impairment is not pronounced in this population.

8.1.1 Intestinal inflammation in EED is triggered by independent mechanisms and does not correlate with intestinal permeability

Elevated levels of fecal NEO or MPO indicate intestinal inflammation, which is one of the key features of EED. This study detected a larger proportion of children who had raised fecal levels of NEO compared to MPO, indicating variation in pro-inflammatory responses. NEO is a low molecular weight molecule belonging to the chemical group known as pteridines. It is synthesized and released upon stimulation of macrophages and dendritic cells with IFN-γ. Thus, elevated levels of NEO in the fecal samples indicate increased activity of macrophages and dendritic cells, which are part of the mononuclear phagocyte system. Both macrophages and dendritic cells are classical antigen-presenting cells; they present antigens to major histocompatibility (MHC) molecules thereby activating

naïve T cells[221]. Additionally, macrophages play a key role in clearing apoptotic cells, cellular debris and pathogens. The prominence of intestinal inflammation indicated by NEO might suggest macrophages and dendritic cells are the primary innate immune response in EED in this setting, which is consistent with the chronic T cell infiltration observed in EED in other populations[3]. Conversely MPO, which is another biomarker of intestinal inflammation, was elevated in only one-third of the children. The differences in the proportion of children with higher fecal levels of MPO and NEO suggest to the candidate that intestinal inflammation in EED is triggered by independent mechanisms.

In serum, AAT makes up the majority of serine protease inhibitors and protects tissues from protease damage during inflammation. AAT is synthesized primarily in the liver but also to a smaller extent in intestinal macrophages, monocytes, and intestinal epithelial cells. Since AAT is relatively resilient to enzymatic digestion, its increased levels in fecal samples indicate intestinal protein loss and increased mucosal permeability. In this thesis, the proportion of children with raised AAT was low suggesting a low proportion of children with a leaky gut. The lack of correlation between fecal markers of intestinal inflammation (NEO or MPO) and AAT suggests that intestinal inflammation in EED does not always result in intestinal permeability.

8.1.2 No evidence of intestinal inflammation and permeability as prominent mediators of growth failure in rural Malawian children

Intestinal inflammation and permeability are some of the key features of EED and there is evidence to support associations between fecal markers of intestinal inflammation or permeability and growth faltering in some populations[53,54,57], however, conflicting evidence also exists. George *et al.* and Campbell *et al.* did not find significant relationships between biomarkers of EED and child growth in low-income settings[56,163], findings that are similar to data reported in this thesis.

The inconsistent data on the relationship between biomarkers of EED and child growth can partially be attributed to differences in study designs. Most of the studies that have reported associations between the biomarkers of intestinal inflammation or permeability and growth were longitudinal studies conducted in children less than 2 years of age and had relatively large sample

sizes[53,57,161]compared to the present study and to other studies that have not found these associations[56,163]. Nonetheless, a study conducted in North-Eastern Brazilian children that had a sample size smaller than the present study (n=375) reported associations between fecal markers of intestinal inflammation or permeability and linear growth in children aged 6 to 26 months[54]. That study found that high fecal levels of MPO and AAT were associated with a reduction in HAZ score of 0.15 at 6-months follow-up. Although the associations between the two biomarkers and HAZ score were statistically significant, a reduction in HAZ score by 0.15 translates to a reduction in height by approximately 0.5 cm[222], which is marginal. Thus, larger sample sizes may still be a prerequisite to detect significant evidence of a relationship between fecal biomarkers of EED and child growth.

Available evidence indicates that the relationship between intestinal inflammation or permeability and child growth is plausible[53,54]. However, EED might not be the primary determinant of growth failure in Malawian children given that associations between intestinal inflammation or permeability and growth impairment have been demonstrated in populations where growth indicators are better compared to Malawi [54,131,223]. The lack of a link between intestinal inflammation or permeability and growth outcomes in Malawian children might also be attributed to the presence of other predominant factors that affect child growth such as poor maternal nutritional status during pregnancy, diarrheal diseases and poor socio-economic status[224-226], which might mask the effect of intestinal inflammation or permeability on growth outcomes.

8.2 Intestinal bacterial carriage and associations with fecal markers of intestinal inflammation or permeability and growth outcomes

Intestinal carriage of *Akkermensia muciniphila, Bifidobacterium longum, Dorea formicigenerans,* and *Faecalibacterium prausnitzii* in rural Malawian children was described using qPCR assay. The associations between intestinal carriage of these bacteria and biomarkers of intestinal inflammation and permeability or growth were also explored. *F. prausnitzii* and *D. formicigenerans* were found to be the most prevalent of the bacteria assayed while *B. longum* was the least prevalent bacterium. *A. muciniphila* was carried by almost half of the children studied. Comparisons of bacterial carriage by age, sex, and area of residence showed significant differences. Intestinal carriage of *B. longum* or *D.*

formicigenerans was associated with biomarkers of intestinal inflammation and permeability. Additionally, intestinal carriage or relative abundance of *B. longum* or *D. formicigenerans* were associated with ponderal growth.

8.2.1 *Intestinal carriage of F. prausnitzii and A. muciniphila was not associated with fecal biomarkers of intestinal inflammation and permeability or growth outcomes*

F. prausnitzii is one of the most common intestinal bacteria whose abundance has been shown to increase with age[176,177]. *F. prausnitzii* has also been identified as one of the most important age discriminatory bacterium in low-income settings[76,168]. This thesis found a high prevalence of *F. prausnitzii.* Additionally, prevalence of *F. prausnitzii* showed a trend that increased with increasing age confirming previously reported trends[177]. However, *F. prausnitzii* carriage did not have any significant effect on biomarkers of EED or growth outcomes.

This study also found carriage of *A. muciniphila* in almost half of the fecal samples tested. *A. muciniphila* is a recently discovered gram-negative bacterium that produces several enzymes that degrade mucin (mucous lining of the gut) subsequently providing carbon and nitrogen to other intestinal bacteria[178]*. A. muciniphila* is said to colonize the intestinal tract early in life and almost reaches the level observed in healthy adults within the first year of life, representing approximately 1 to 3% of the total microbiota[179]*.* The abundance of *A. muciniphila* has been strongly associated with markers of lipid metabolism and negatively associated with inflammation in adipose tissue in a mouse model [174] while in adult humans, the presence of *A. muciniphila* has shown an inverse association with body weight[175,227,228]. A study that was conducted to understand the physiological roles of *A. muciniphila* in obesity reported an increase in the expression of markers of antimicrobial peptides in *A. muciniphila*-treated mice indicating potential antimicrobial effects[229]*.* In the present study*, A. muciniphila* carriage was observed in almost half of the children but was not associated with fecal biomarkers of intestinal inflammation, permeability or child growth, therefore, the significance of *A. muciniphila* carriage in this population needs to be further investigated.

8.2.2 *Area of residence was associated with intestinal inflammation or permeability and prevalence of A. muciniphila carriage*

This thesis found associations between geographic location and fecal levels of AAT and MPO. Fecal samples of children from Namwera had higher levels of AAT and MPO compared to the other 4 geographic locations. The higher levels of fecal biomarkers shown in children from Namwera could be explained by geographical and / or climatic factors that subsequently contribute to poor sanitation and hygiene. Namwera lies at a higher altitude compared to Mangochi, Monkey Bay, Makanjira and Chilipa and generally experiences more rainfall compared to the other 4 locations[230,231]. Higher rainfall allows rapid spread of enteropathogens[232] whose carriage in children from similar settings has been associated with raised AAT, MPO and NEO[57].

This thesis also found differences in the prevalence of *A. muciniphila* carriage by geographic location. A smaller proportion of children who resided in Mangochi carried *A. muciniphila* compared to children from the other 4 locations. Mangochi is a peri-urban setting compared to the other 4 rural locations. Children in Mangochi might have had access to cleaner water and improved sanitation compared to the rest of the children. Nonetheless, this thesis did not assess the socio-economic status of these children as such, the candidate can only postulate that socio-economic status affects intestinal carriage of *A. muciniphila*.

8.2.3 *Unexpected association between fecal carriage of B. longum and raised fecal markers of EED or lower WAZ scores in Malawian children*

In this thesis, *B. longum* was the least prevalent intestinal bacterium of the four assayed and its carriage was primarily found in fecal samples of children aged between 1 and 12 months. This supports data from previous studies that indicated early colonization of *B. longum* in the human gut [64,68,181]. Since *B. longum* is more abundant during the first 18 months of human life[181], the lower proportion of fecal samples positive for *B. longum,* in comparison to other studies, likely reflects the small number of young infants who were included in this study. Less than one-third of the children included in this study were ≤12 months old. *B. longum* carriage was also associated with elevated levels of fecal AAT, MPO,

and NEO. Correspondingly, fecal levels of AAT, MPO, and NEO were elevated in children aged between 1 and 12 months compared to older children in this thesis. The higher abundance of *B. longum* in younger infants has been attributed to breastfeeding; *B. longum* is said to be present in human breast-milk[182,183]. Additionally, breastfeeding has been associated with high fecal levels of AAT and MPO in low-income settings[161]. Therefore, the association between *B. longum* carriage and fecal levels of AAT, MPO, and NEO could be due to residual confounding by breastfeeding and not necessarily an effect of *B. longum* carriage. Breastfeeding status was not assessed in this thesis. Adjusting for age in the analyses should have mitigated for confounding by breastfeeding given the association between age and breastfeeding. However, age stratified analysis on the associations between *B. longum* carriage and the biomarkers showed significant associations for the age groups less than 2 years highlighting a potential effect of breastfeeding; most children of this age group consume breastmilk in this population[131].

Intestinal carriage of *B. longum* was associated with lower WAZ scores while the increased relative abundance of *B. longum* was associated with better WHZ scores. The relationship between *B. longum* carriage and lower WAZ score is surprising considering that *B. longum* is widely associated with good health. To date, there is no available data indicating the relationship between the presence or absence of *B. longum* in the gut and ponderal growth in humans. Considering that there was a positive relationship between the relative abundance of *B. longum* and WHZ scores, the candidate suggests that analyses focusing on just absence or presence of bacteria can yield different results from analyses that take into account bacteria load. Moreover, Subramanian *et al.* found a lower relative abundance of the genus *Bifidobacterium* in Bangladeshi children with poor WHZ compared to children with better WHZ[168], which is consistent with the relationship between the relative abundance of *B. longum* and WHZ reported in this thesis. These results, therefore, suggest a potential role of *B. longum* in ponderal growth and offers insight on the discrepancies different analyses might have. The lack of firm evidence on the effects of *B. longum* carriage or relative abundance on growth in the human population leaves room for uncertainty as such the association between *B. longum* carriage and WAZ should not be ignored but rather investigated further.

8.2.4 *Intestinal carriage of D. formicigenerans is potentially associated with a healthy gastrointestinal tract*

D. formicigenerans was reported as the second most prevalent bacterium of the bacteria tested in this thesis and its carriage was higher in older children than younger children. *D. formicigenerans* is a gram-positive obligate anaerobic bacterium that is known to produce large amounts of formic acid from carbohydrate fermentation[233]. Formic acid is one of the organic acids whose supplementation in poultry feeds has been associated with beneficial effects on poultry performance, immunity and gut health via reduction of intestinal bacterial infections[234,235]. The high prevalence of *D. formicigenerans* carriage in rural Malawian children and its association with normal fecal levels of AAT, MPO, and NEO indicate the potential of this bacterium to have anti-inflammatory effects in the human gut and also suggests other beneficial effects this bacterium might have on gastrointestinal health.

8.3 The gut microbiota of children with signs of EED differs from that of children without these signs

The gut microbiota in children with and without signs of EED was characterized through sequencing of the bacterial 16SrRNA gene and quantification of the biomarkers of EED in fecal samples. There was no association between gut microbiota diversity and AAT or MPO nor was there a relationship between gut microbiota diversity and composite EED score. However, there was a negative association between fecal microbiota diversity and fecal concentration of NEO. Comparison of bacterial community composition between normal and elevated biomarker concentration showed no significant differences, but significant differences were found between low and high composite EED score. These results highlight the potential role of the gut microbiota in EED development.

8.3.1 *Increase in microbiota diversity is associated with reduced intestinal inflammation*

To date, there is no data available supporting the relationship between fecal microbiota diversity and intestinal inflammation in EED. However, available evidence from IBD studies suggests an association between intestinal inflammation and reduced intestinal microbiota diversity[213,236,237], which is similar

to what has been reported in this thesis. These results, therefore, show consistency in the relationship between increased fecal microbiota diversity and reduced intestinal inflammation suggesting a potential role of the gut microbiota in modulating inflammatory responses. In this thesis, an increase in gut microbiota diversity was associated with low levels of NEO, which suggests that high microbiota diversity modulates innate immune responses by downregulating macrophage or dendritic cell activity.

Findings from this thesis support existence of a link between gut microbiota diversity and intestinal inflammation that has also been reported in IBD studies. However, EED and IBD are different conditions and therefore the mechanisms behind such a link should be different for the two conditions. In IBD, there has been a debate regarding what comes first between changes in gut microbiota diversity or intestinal inflammation. Sepehri and colleagues suggested that the reduction in microbiota diversity is a consequence of intestinal inflammation triggered by enteropathogens[238]. This suggestion would, therefore, indicate that gut microbiota diversity increases in the absence of enteropathogens.

8.3.2 The intestinal bacterial community composition in children with low and high composite EED scores is different

There is limited data indicating associations between intestinal bacterial community structure and EED. Recently, Ordiz *et al.* found differences in intestinal bacterial composition between children with increased and normal levels of intestinal permeability measured by the L:M ratio test[59]. At phylum level, *Proteobacteria* was significantly reduced in children with increased intestinal permeability compared to children with normal levels while prevalence of genera *Megasphaera*, *Mitsuokella*, and *Sutterella* was higher in children with increased intestinal permeability compared to children with normal levels. *Succinivibrio*, *Klebsiella*, and *Clostridium_XI* were less prevalent in children with increased intestinal permeability compared to children with normal levels. Similarly, this thesis found an association between higher abundance of *Succinivibrio* and low composite EED score. To date there are no other studies that have characterized the gut microbiota in EED using the composite EED score. The consistency in the association between *Succinivibrio* abundance and EED indicates the potential role of *Succinivibrio* in improving EED.

Succinivibrio is a gram-negative anaerobic commensal of the human colon that is normally present in low abundance[239,240]*. Succinivibrio* is a genus of phylum Proteobacteria and has two species*, Succinivibrio amylolytica,* and *Succinivibrio dextrinosolvens.* The abundance of these species differs depending on the diet. *S. amylolytica* is found in high abundance in the rumen of livestock that are fed diets consisting of hay and grain while *S. dextrinosolvens* is found in higher abundance in the rumen of livestock fed a starch-rich diet or other diets that include large quantities of fermentable carbohydrates[241,242]. *Succinivibrio* has not been extensively studied but its higher abundance in the bovine rumen has been associated with fiber-degradation[214]. Interestingly, high abundance of this bacterium has been shown in the fecal microbiota of rural African children, consuming a diet rich in fiber and non-animal protein[88] emphasizing the role of this bacterium in fiber degradation. Fiber degradation by anaerobic bacteria produces SCFAs whose supplementation in poultry feeds has been associated with a reduction of intestinal bacterial infections[234,235]. Drawing from this, the candidate hypothesizes that the higher abundance of *Succinivibrio* in the gut inhibits colonization by enteropathogens thereby improving signs of EED. Thus, Succinivibrio is potentially beneficial to gut health*.*

8.4 Long-term treatment with azithromycin was weakly associated with *Prevotella* abundance

This study assessed the long-term impact of azithromycin treatment on the fecal microbiota of Malawian children. Azithromycin treatment did not have an impact on gut microbiota diversity, however, there was a higher abundance of *Prevotella* at 24-months in azithromycin recipients indicating long-term effects of azithromycin treatment on the gut microbiota composition.

Prevotella is a gram-negative commensal bacterium found on the mucosal sites of the respiratory tract, the gut, and the oral cavity. The genus *Prevotella* is a member of phylum *Bacteroidetes*, which includes other clinically relevant genera such as *Bacteroides* and *Porphyromonas*. Although emerging evidence from murine studies has implicated *Prevotella copri* in experimental colitis[243], reduced relative abundance of *Prevotella* has been reported in pediatric Crohn's disease in humans[220]. Drawing from the latter finding, together with the findings of this thesis, the candidate suggests that the increase in the abundance of *Prevotella*

following long-term azithromycin treatment is potentially beneficial to human gut health. Interestingly, these findings are not consistent with findings by Korpela *et.al.* who reported increased abundance of phylum *Bacteroidetes,* to which *Prevotella* belongs*,* in children who had used macrolide antibiotics within 6 months of sample donation but found no impact in children who had used the antibiotics longer than 6 months[127]. This inconsistency suggests that either the long-term impact of azithromycin treatment on phyla and genera are different or that the long-term effect of azithromycin treatment on gut microbiota composition varies depending on other factors, such as geography and socio-economic status. The study by Korpela *et al.* was conducted in a high-income European setting.

8.5 Limitations

8.5.1 Reference values for normal or raised fecal biomarkers of EED in low-income settings and the composite EED score

Reference values of fecal AAT, MPO, and NEO in low-income settings have yet not been established, as such the adoption of Western values was the only available option. However, the available Western reference values reported in the literature for some of the fecal biomarkers have been determined from adult IBD studies[244]. IBD is strikingly a different condition from EED and fecal levels of these markers in adults might be different from children as trends of these markers have been shown to decrease with increasing age[161]. Also, the trends of these biomarkers in Western populations might vary from populations in low-income settings. Therefore, the adoption of the Western reference values in this study might have undermined or exaggerated the magnitude of intestinal inflammation or permeability reported in this thesis. However, this thesis did not find associations between the uncategorized concentration values and growth suggesting that the use of these reference values might not have adversely affected the association between categorized fecal biomarkers of EED and child growth in this thesis.

This study also used the composite EED score to indicate EED in chapters 4, 5 and 6. Despite the significant associations the composite EED score has shown with growth in the previous studies[53,56], the use of the composite EED score has yet to be validated. Therefore, the associations between fecal microbiota and

composite EED score reported by this study might not be a true reflection of what occurs in EED. However, the observation that high abundance of *Succinivibrio* was associated with low EED score in this thesis is consistent with findings from a recent Malawian study that found a higher abundance of *Succinivibrio* in children with normal levels of intestinal permeability[59]. This conformity somewhat underscores the validity of the composite EED.

8.5.2 Use of commercial kits to describe the intestinal bacterial carriage

Chapter 5 described intestinal carriage of *B. longum, D. formicigenerans, A. muciniphila, F. prausnitzii* and explored their associations with growth or fecal biomarkers of EED. Use of commercial qPCR kits to describe the prevalence of bacterial carriage limited the number of bacterial targets studied, as primers and probes for other targets that could have been included in the study were not commercially available. *Clostridium butyricum* for example is one bacterium that has been associated with growth in a mouse model and its inclusion in the study would have been valuable, but its qPCR assay was not commercially available. Nevertheless, using the few targets that were available, this study showed associations between individual bacteria and biomarkers of EED or child growth suggesting that commercial assays can be conveniently used to study intestinal bacteria in settings where bacterial 16S rRNA gene sequencing platforms cannot be easily accessed.

The lack of standards in the available commercial qPCR kits made absolute quantification of each bacterium impossible. Absolute bacteria quantification would allow comparative analyses of total number of bacteria versus absence or presence of bacteria. However, the use of the published bacterial quantification PCR assay allowed relative quantification of each bacterium and offered insight into the differences that may be present in an analysis that is centered on the relative number of bacteria versus analysis that focuses solely on the presence or prevalence of the bacterium.

8.5.3 Lack of data on risk factors for EED

This study opportunistically utilized fecal samples from a study that was designed to investigate the impact of azithromycin on childhood morbidity and as such did

not have data on other factors that influence fecal levels of AAT, MPO and intestinal bacterial colonization such as data on breastfeeding status, access to water, sanitation and hygiene. Human breast milk has been associated with high levels of fecal AAT and MPO[161] and a higher abundance of *Bifidobacterium*[191]. The relationship between breastfeeding and higher levels of fecal MPO or AAT or high abundance of *Bifidobacterium* can be attributed to the presence of the biomarkers[164] or *Bifidobacterium* in the human breast milk[182,183]. The higher levels of AAT, MPO, and NEO observed in younger children compared to older children in this thesis indicate that breastfeeding might have contributed to the higher levels of fecal AAT, MPO and NEO observed in young children since most Malawian children, like children in other populations, consume maternal breast milk together with other complementary foods into the second year of life[131]. Therefore, the absence of data on the breastfeeding status of the children included in this study might have led to residual confounding resulting in the unusual associations between intestinal carriage of *B. longum* and raised AAT, MPO or NEO. Adjusting for breastfeeding status in analyses involving fecal levels of AAT, MPO and NEO has been previously recommended[161]. However, given the clear association between breastfeeding and age, adjusting for age in the analyses should have mitigated for potential confounding by breastfeeding status.

Access to potable drinking water, living in poor sanitary conditions and consumption of unhygienic foods are some of the suggested risk factors for EED[94]. A recent study in South-Western Uganda reported an association between unsafe drinking water and EED in young children. Children from households with unsafe drinking water had higher levels of intestinal permeability compared to children from households with safe drinking water[245]. Another study that assessed the relationship between environmental contamination and EED in rural Bangladeshi children reported associations[14]. Here, children from clean households had lower levels of intestinal permeability and microbial translocation compared to children from contaminated household environment. Additionally, children from clean households had a lower prevalence of parasites tested compared to children from contaminated household environment. Parasites, which are some of the suggested exposures in EED etiology, have been associated with biomarkers of EED and growth faltering in low income settings[57].

The lack of data on access to safe drinking water, improved sanitation and hygienic practices limited a more informative analysis that could have been conducted to better explore some of the associations reported in this study.

8.5.4 Use of 16S rRNA gene sequencing

One challenge in 16S rRNA gene sequencing approach is the presence of contaminants introduced either during sample collection or during processing in the laboratory. It is usually recommended as good practice to include reagent-only controls during 16S rRNA gene sequencing processes as a way to check reagent contamination. In this thesis, the extraction controls for each batch of DNA isolation reagents used and environmental controls were included during the isolation of DNA from the samples. Non-template controls were also included during library preparation. The controls were sequenced together with the samples and bacterial sequences were detected in the controls. The presence of bacterial sequences in the controls is not surprising as commercial reagents (extraction kits, primers, and polymerases) are known to contain microbial DNA contaminants, which vary in amounts from one batch to another[246] and common laboratory contaminants have been reported to be present in processed samples[247]. To date, there is no consensus on how to deal with sequences identified as environmental or reagent contaminants. Some authors suggest subtracting the “contaminant” sequences, but this is not appropriate as some of the OTUs found in the controls can genuinely be found in samples, therefore, their removal can affect the interpretation of the data.

To reduce the impact of potential contaminants on fecal microbiota diversity and composition, samples with >1000 total reads were retained and from these, only bacterial OTUs identified to the genus level, with sequences more than 0.005% of the total number of sequences[143] and a frequency of more than 0.01% in any sample were kept. Additionally, OTU data was rarefied to 1000 reads with 1000 permutations before calculating diversity indices and determining bacterial community differences between samples or was normalized by cumulative sum scaling (CSS) before performing univariate analysis with individual taxa[144,145].

Another challenge with partial-full length 16S rRNA gene sequencing is that its resolution is generally limited to the genus level making it impossible to perform comprehensive microbiota analyses at the species level, which would be more

informative. Additionally, 16S rRNA gene sequencing as used here targets a limited number of variable segments of the 16S gene that are present in bacteria and archaea. As such, even full length 16S rRNA gene sequencing is not representative of all constituents of the microbiota such as viruses, protozoa and fungi. Whole genome shotgun sequencing is an alternative sequencing approach that has greater taxonomic resolution, and the ability to infer functional profiling and cross-domain coverage, however, this sequencing approach is more expensive and requires greater computational power and analysis.

8.5.5 Study design

This PhD project utilized a platform provided by the MORDOR-Malawi study, which was designed to test hypotheses different from this PhD thesis. The fecal samples, demographic and anthropometric data available to this PhD project, through the MORDOR-Malawi platform, were collected cross-sectionally. Some of the challenges with cross-sectional studies are that they do not help with evaluating cause and effect and, the timing of a sampling is not assured to be representative. Nonetheless, this thesis reported associations some of which are consistent with findings reported by longitudinal studies.

As this PhD thesis used fecal samples originally collected for other purposes, the sample volumes remaining to this PhD project were not always sufficient to carry out all the desired tests. As such, some samples were tested for all the biomarkers while other samples were only tested for one or two biomarkers giving rise to missing data. Missing data can introduce bias, make data handling and analysis more difficult, and can reduce power. One way of alleviating problems caused by missing data is conducting data imputation. However, data imputation can reduce variance and distort relationships between variables, and was not done for this PhD thesis. Another limitation that arose from the opportunistic use of MORDOR-Malawi fecal samples and data was the smaller sample sizes available for various objectives. Smaller sample sizes might have lowered the statistical power to establish associations between biomarkers and growth outcomes, or to detect pronounced differences in intestinal microbiota between treatment groups.

8.6 Future work

8.6.1 *Understanding the significance of D. formicigenerans, Succinivibrio, and Prevotella in gut health*

Future studies should explore the significance of intestinal carriage of *D. formicigenerans, Succinivibrio* and *Prevotella* and other potentially beneficial bacteria such as *Bifidobacterium* in humans. The studies should aim to demonstrate whether intestinal carriage of these bacteria protects against colonization by enteropathogens thereby reducing intestinal inflammation or permeability. PCR assays that can test a panel of up to 40 enteropathogens in a single sample and individual PCR assays for some of the putatively beneficial bacteria are commercially available, therefore, conducting such studies should not be very challenging. Such studies would uncover potential probiotic candidates for EED therapy in addition to providing knowledge on the specific enteropathogens that need to be targeted by EED interventions.

In vitro and *in vivo* studies can also be used to understand the specific mechanisms through which the abundance of *D. formicigenerans, Succinivibrio* and *Prevotella* influence gut health. These bacteria are commonly found in the colon of populations consuming a plant-rich diet, a good source of fiber. Fiber-degradation by anaerobic bacteria in the colon produces SCFAs. Previous studies of probiotics and studies of organic acid supplementation in animals have utilized colonic or epithelial cell-lines to understand the effect of certain bacteria on intestinal inflammatory responses or gut health in general. Future studies could therefore isolate strains of *D. formicigenerans, Succinivibrio*, *Prevotella*, *Bifidobacterium* and culture these isolates in colonic or epithelial cell-lines colonized with enteropathogens to explore whether the metabolites produced from fiber-degradation promote gut integrity by preventing enteropathogen colonization via direct killing of enteropathogens or by out-competing pathogenic bacteria for nutrients. Such studies could also demonstrate if different strains of the beneficial bacteria have varying effects on gut health.

8.6.2 *Establishing reference values for fecal AAT, MPO, and NEO in low-income settings*

To develop reference values for biomarkers of EED, studies would need to recruit children from different geographic regions representing low-income settings.

Recently, a multi-site study was implemented to investigate associations between EED and growth in infants from low-income settings of South Asia, Latin America, and Sub-Saharan Africa[248]. This study measured fecal levels of AAT, MPO, and NEO. The data collected by this study, therefore, presents an opportunity to develop reference values for fecal AAT, MPO, and NEO in children living in low-income settings.

8.6.3 Validity of the composite EED score

The composite EED score has been previously shown to predict linear growth deficits in infants[53]. This thesis found an association between low composite EED score and higher abundance of *Succinivibrio*, which is consistent with recent findings that used different biomarkers of EED[59] underscoring the potential of the composite EED score to indicate EED disease activity. Nevertheless, the use of this score in EED has not been properly validated. Therefore, future studies should aim to validate the composite EED score using "gold standard" tests. Endoscopy or biopsy would be the ideal tests to use to validate this score, however, these tests are invasive as such cannot be implemented in asymptomatic participants. L:M ratio tests, which have correlated well with histopathologic changes in EED, could therefore be used as an alternative to validate the use of this score. Studies should also assess if the composite score is optimal with only the three biomarkers (NEO, MPO, and AAT) or if markers of other processes of EED can be incorporated in the score. Alpha-1 acid glycoprotein (AGP) is a marker of systemic inflammation that has been strongly associated with growth faltering in low-income settings[57] as such its incorporation into the composite EED score could improve the utility of the score.

8.7 Summary

Almost two-thirds of rural Malawian children had elevated NEO, while MPO was raised in one-third of the children and AAT was elevated in less than one third of the children making it the least elevated biomarker, of those tested, in this population. Consistent with previous findings, biomarker levels were significantly higher in younger children compared to older children. There was no association between biomarkers of EED (individually or as a composite score) and growth impairment suggesting that EED is not the main driver of growth impairment in this population.

F. prausnitzii and *D. formicigenerans* were found to be the most prevalent bacteria, of those assayed for, in children; *F. prausnitzii* was present in almost all fecal samples while *D. formicigenerans* was present in over two-thirds of the fecal samples tested. Both bacteria were more prevalent in older children compared to younger children, which is similar to previous findings. In contrast, *B. longum* was present in just over one-third of the fecal samples making it the least prevalent bacteria assayed. Additionally, intestinal carriage of *B. longum* differed by age; higher prevalence of the bacterium was observed in younger children compared to older children. Intestinal carriage and relative abundance of *B. longum* were associated with elevated individual biomarker concentration and high composite EED scores, which is unusual. Additionally, intestinal carriage of *B. longum* was associated with lower WAZ while increased relative abundance of this bacterium correlated with an increase in WHZ suggesting a potential role of individual bacteria in ponderal growth in humans. Intestinal carriage of *D. formicigenerans* was associated with normal biomarker concentration suggesting a beneficial role of this bacterium in gut health.

Fecal microbiota diversity was weakly associated with reduced fecal concentration of NEO indicating a link between increased fecal microbiota diversity and reduced intestinal inflammation. There were significant differences in fecal microbiota composition between low and high composite EED scores suggesting perturbations in overall gut microbiota composition in EED. Azithromycin treatment was not associated with fecal microbiota diversity, but was weakly associated with relative abundance of *Prevotella* at 24-months follow-up suggesting potential long-lasting effects of azithromycin on the abundance of defined bacterial taxa but not overall microbiota diversity.

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10. Appendix

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Observational / Interventions Research Ethics Committee

Robin Bailey
CR / ITD
LSHTM

17 December 2013

Dear Professor Bailey,

Study Title: **Mortality Reduction After Oral Azithromycin in Malawi (MORDOR-Malawi)**
LSHTM ethics ref: **6500**

Thank you for your email of 12 December 2013, responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	2	
Protocol including Information Sheets & Consent forms	2	10/12/2013

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form. All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via form E4. An annual report form (form E3) is required on the anniversary of the approval of the study and should be submitted during the lifetime of the study. At the end of the study, please notify the committee via form E5.

[redacted]

Professor John DH Porter
Chair
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http://www.lshtm.ac.uk/ethics/

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.02/14/1521 – Mortality Reduction after Oral Azithromycin in Malawi (MORDOR-Malawi) by Dr. K. Kalua

On 17 March 2014

As you proceed with the implementation of your study, we would like you to adhere to international ethical guidelines, national guidelines and all requirements by COMREC as indicated on the next page

[redacted]

Dr. V [redacted] C)

Approved by
College of Medicine
17 MAR 2014
(COMREC)
Research and Ethics Committee

17/03/14
Date

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Observational / Interventions Research Ethics Committee

Mr David Chaima
LSHTM

13 September 2017

Dear Mr David Chaima,

Study Title: Assessing the impact of azithromycin on biomarkers of environmental enteric dysfunction and linear growth in Malawian children

LSHTM ethics ref: 14280

Thank you for your application for the above research, which has now been considered by the Observational Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Protocol / Proposal	MORDOR ethics approval LSHTM	17/12/2013	2
Protocol / Proposal	MORDOR trial protocol	10/03/2014	2
Local Approval	Screen shot Higher training in MORDOR	10/03/2014	2
Information Sheet	Consent form - OBJECTIVE 5_English	14/03/2014	1
Information Sheet	Consent form - OBJECTIVE 5_Yao	14/03/2014	1
Information Sheet	Consent forms - OBJECTIVE 5_Chichewa	14/03/2014	1
Information Sheet	Information sheet - OBJECTIVE 5_Chichewa	14/03/2014	1
Information Sheet	Information sheet - OBJECTIVE 5_English	14/03/2014	1
Information Sheet	Information sheet - OBJECTIVE 5_Yao	14/03/2014	1
Local Approval	COMREC MORDOR Approval 17-03-2014	17/03/2014	1
Protocol / Proposal	Anthropometry measurements Electronic form	01/05/2014	NA
Protocol / Proposal	Census individual Electronic form	01/05/2014	NA
Protocol / Proposal	Stool sample collection Electronic form 1	01/05/2014	NA
Protocol / Proposal	Stool sample collection Electronic form 2	01/05/2014	NA
Protocol / Proposal	Verify consent Electronic form	01/05/2014	NA
Protocol / Proposal	Intestinal permability Clinic form Baseline	12/04/2015	3
Protocol / Proposal	Intestinal permability Clinic form 12 weeks	12/04/2015	3
Protocol / Proposal	Census household Electronic form	01/05/2015	NA
Information Sheet	Consent - Objective 7 ENGLISH	07/09/2015	2
Information Sheet	Consent - Objective 7 YAO	07/09/2015	2
Information Sheet	Information sheet - Objective 7 CHICHEWA	07/09/2015	2
Information Sheet	Information sheet - Objective 7 ENGLISH	07/09/2015	2
Information Sheet	Information sheet - Objective 7 YAO	07/09/2015	2

Protocol / Proposal	Intestinal permability Clinic form 2 weeks	12/04/2016	3
Investigator CV	Maleta Biosketch 2017-01-12	12/01/2017	2017-01-12
Investigator CV	BURR Sarah CV Mar 2017	01/03/2017	March 2017
Investigator CV	CV_David Chaima_19-06-2017	19/06/2017	19-06-2017
Investigator CV	Dr Khumbo Kalua CV	21/06/2017	June 2017
Investigator CV	Prof Bailey CV	21/06/2017	Jun 2017
Investigator CV	F-CV Template - Martin J Holland - 2017	22/06/2017	June 2017
Protocol / Proposal	Access to MORDOR data from trial coordinator	28/06/2017	NA
Protocol / Proposal	Access to MORDOR data from PI	11/07/2017	NA

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

[redacted]

Professor John DH Porter
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

COLLEGE OF MEDICINE RESEARCH AND ETHICS COMMITTEE

Request for Amendment/Modification

Please complete the following:

COMREC REF. Number: P.02/14/1521 (COMREC will not process requests without this number.)	**Date of Request: 21 November 2017**
Principal Investigator Name: Prof. Khumbo Kalua Phone # : 0999958176 Email: kkalua@medcol.mw khumbokalua@yahoo.com	**Contact Person** (if other than PI): **Virginia Mlenga** Phone #: 0994146522 Email: virginia@bicomalawi.org
Title of Study: **Childhood Mortality Reduction after Oral Azithromycin in Malawi (MORDOR- Malawi)**	

1. **Description of proposed changes:** (Note: Changes may not be implemented before COMREC approval)

   Use attachments and additional pages, as needed. The proposed changes should be reflected in the approved protocol.

   i) We request that all stool samples collected by the MORDOR study be tested for the following markers of intestinal inflammation: neopterin, calprotectin, myeloperoxidase and alpha-1-antitrypsin by ELISA, and for the presence of bacterial pathogens by real-time PCR. All testing will be conducted in the MORDOR lab in the microbiology building at CoM.

   ii) We request that the microbiome studies, already described within the approved MORDOR protocol, be conducted in collaboration with Prof Martin Holland of London School of Hygiene and Tropical Medicine and Prof Rob Knight of University of California, San Diego.

   iii) We request that an additional aliquot of bacteria isolates collected by the MORDOR study be archived at Malawi Liverpool Wellcome trust. This would be in addition to the aliquot currently being stored at CoM.

2. **Reason for Amendment/Modification:**

i) As a part of the agreement between the College of Medicine (CoM) and London School of Hygiene and Tropical Medicine (LSHTM), we have offered a programme of PhD study to Mr. David Chaima, the scientific officer employed on the MORDOR trial. Mr. Chaima is now registered as a part-time graduate student at LSHTM and as a visiting student at

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Version: #1:0 **Effective date: 25th November, 2007**

Most of the work comprising Mr. Chaima's proposed PhD project was built into the original MORDOR protocol, under objectives 6 (to assess the effect of mass azithromycin treatments on linear growth in Malawian children) and 7 (to assess the impact of azithromycin on growth faltering mediated via intestinal inflammation and permeability.). However, a part of the LSHTM's requirement for a PhD candidate is that they bring to their research project some aspect that was not previously planned by the project supervisors. This helps the candidate demonstrate his/her ability for independent research and knowledge of the current literature. To this end, Mr. Chaima is interested to use samples and data collected by the MORDOR trial to answer questions surrounding the associations between bacterial pathogens, intestinal inflammation and growth faltering. These are:

1. What biomarkers of intestinal inflammation are associated with stunting (defined as a height-for-age Z score less than 2 standard deviations below the mean) in children residing in rural Malawi?
2. Can these biomarkers be used to predict risk of subsequent growth faltering in Malawian children?
3. Are bacterial pathogens in the gastrointestinal tract associated with biomarkers of inflammation?

To aid Mr. Chaima with his studies, we therefore request approval to amend the MORDOR-Malawi protocol as described above (section 1, part i). These modifications do not require any new samples be collected; Mr. Chaima would conduct the additional testing on the samples already been collected under Objective 5 of the trial, which is to collect stool samples to monitor the emergence of macrolide resistance in gastrointestinal pathogens and Objective 7, which is to assess the impact of azithromycin on growth faltering mediated via intestinal inflammation and permeability.

The proposed amendments are highlighted in the modified protocol attached here as Appendix One. Mr. Chaima's name has been added as an investigator on page 2. The additional ELISA assays to be conducted on the samples are given on pages 32 and 33. All ELISA assays will be conducted at the College of Medicine as indicated on page 35. The additional real time PCR tests are indicated on page 33. Real time PCR assays will be conducted at the College of Medicine as indicated on page 35. The currently approved protocol is attached here as Appendix Two. Mr. Chaima's PhD proposal is attached here as Appendix Four.

ii) In the original MORDOR protocol, we indicated microbiome studies would be done to characterize the effect of azithromycin treatment on the microbial diversity of the normal bacterial flora of the body. However, at the time the original protocol was written, we had not yet identified an academic partner to assist us with this work and we instead indicated that we would inform COMREC as soon as a suitable partner had been found. We are now

asking for permission to conduct these studies with Prof Martin Holland of the London School of Hygiene and Tropical Medicine (LSHTM) and Prof Rob Knight of the University of California, San Diego (UCSD). Prof Martin Holland is an immunologist with extensive experience in genomics, and is a supervisor for David Chaima's PhD. Prof. Rob Knight is a leading authority on the microbiome and has previously collaborated with the College of Medicine in work characterizing the microbiome of Malawian children with kwashiorkor.

Given the complexity of microbiome data and the vast array of methods available for its analysis, we are proposing to conduct the microbiome work through two work streams – one at the LSHTM and the second at the UCSD. Both groups will conduct data analysis independently and the final results will be compared. If the findings of one analysis are real and significant, they should be detected by the second analysis. We feel this parallel approach is important in ensuring the integrity of the results.

Samples will be sent to LSHTM and UCSD for whole genome and 16S pyrosequencing as indicated on pages 33 and 35 of Appendix Two attached. Intestinal microbiome work will be incorporated into David Chaima's PhD and he will be provided the opportunity to travel to London to learn how to conduct these studies. Confirmatory real time PCR, to verify the presence of specific bacteria in stool samples, will be conducted at the CoM in Blantyre as a part of Mr. Chaima's PhD, as explained above. A Material Transfer Agreement with LSHTM is attached here as Appendix Three. A Material Transfer Agreement with UCSD is pending and will be sent to COMREC as soon as it is available. The LSHTM will bear the cost of all sample shipment and processing.

iii) Bacterial isolates generated to assess the effect of azithromycin on carriage of antibiotic resistant bacteria are being stored at CoM storage for the duration of the MORDOR trial. However we have had issues with power fluctuations in the CoM lab, which could potentially undermine the viability of these isolates. In order to safe-guard these samples, we are requesting COMREC permission to store a second aliquot of all bacterial isolates in the MLW archive

The proposed amendments are highlighted in the modified protocol attached here as Appendix One. Storage of the bacterial isolates at MLW is described on page 31 of the modified protocol. The currently approved protocol is attached here as Appendix Two.

3. Changes to Consent Form: Are changes required? No X_____ Yes _____ (If Yes, attach new consent form).

[redacted]

Signature of Principal Investigator

21 November 2017_
Date

COMREC Office Use only:
Approval
date:_____

Approved by:
_____

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Recommended : ✓ *
Not recommended : _____

Signature [redacted] Date 13 December 2017

IRB Chairperson or Authorized Signatory

Dr YB Mlombe

COMREC form 103

*Approval recommended on condition that COMREC contact details are updated since Dr Manda-Taylor is no longer with COMREC.

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